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Clinical and Experimental

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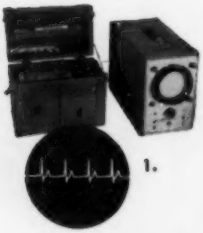

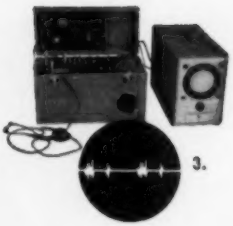

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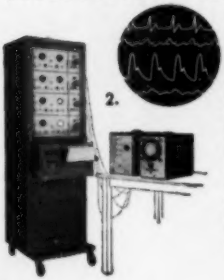

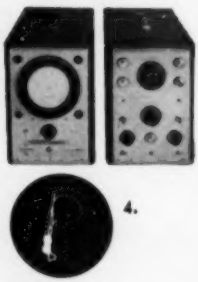

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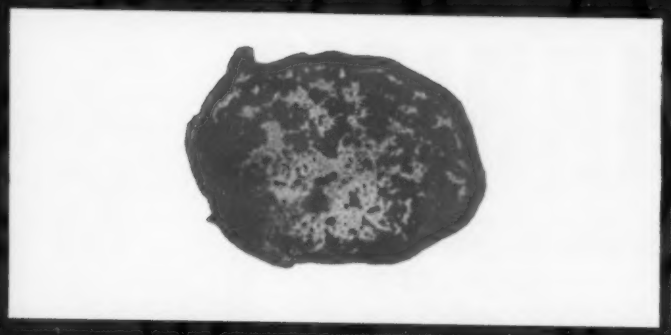

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***Peterson, E. T., Walford, R. L., Figueroa, W. G. and Chisholm, R. Ox Cell Hemolysins in Infectious Mononucleosis and in Other Diseases. Am. J. Med. *21(2):* 193-199 (Aug.) 1956. (Hyland Laboratories will send you a reprint of this article promptly, at your request.)**

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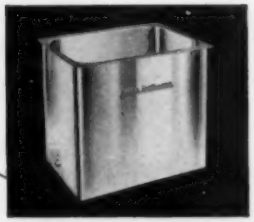

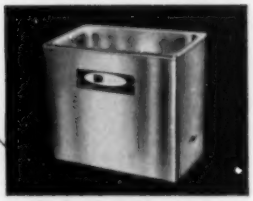

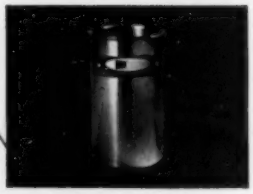

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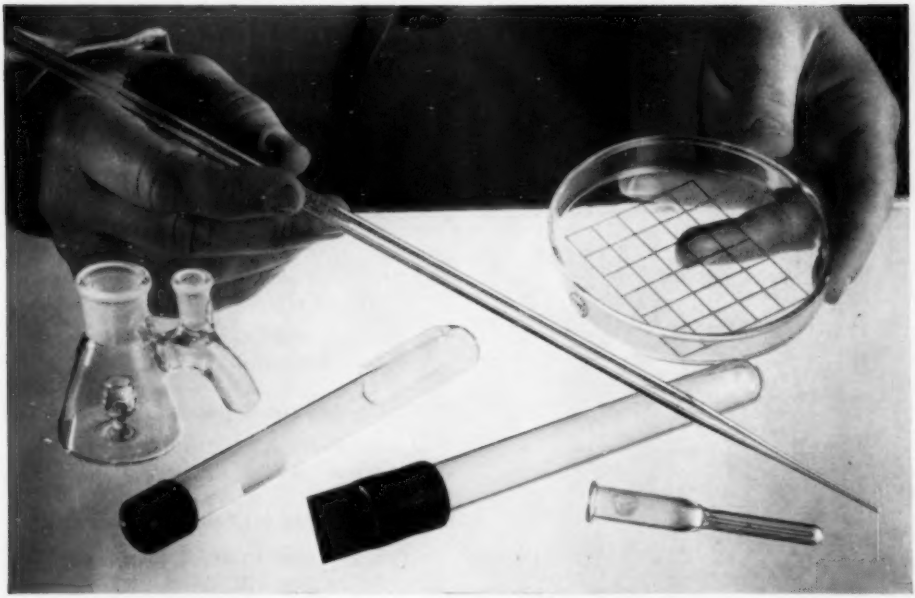

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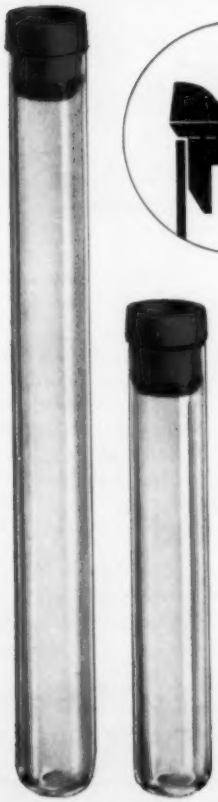

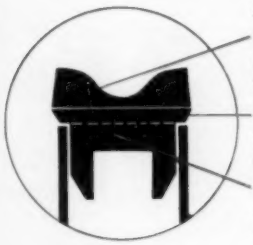

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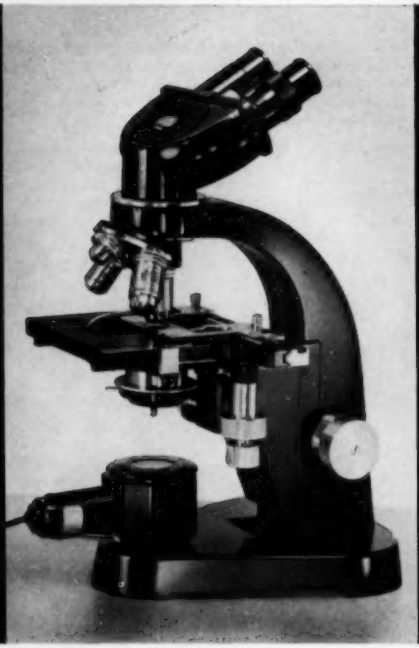

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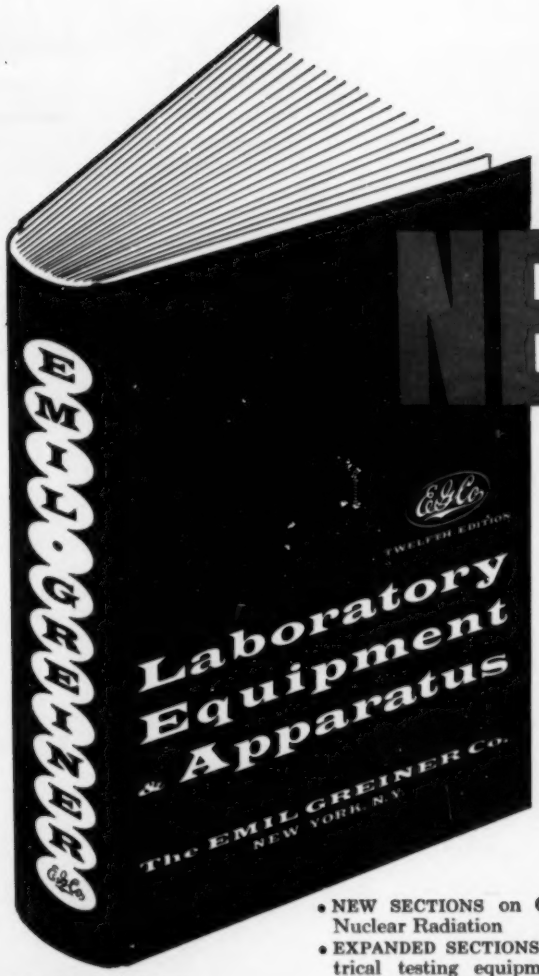

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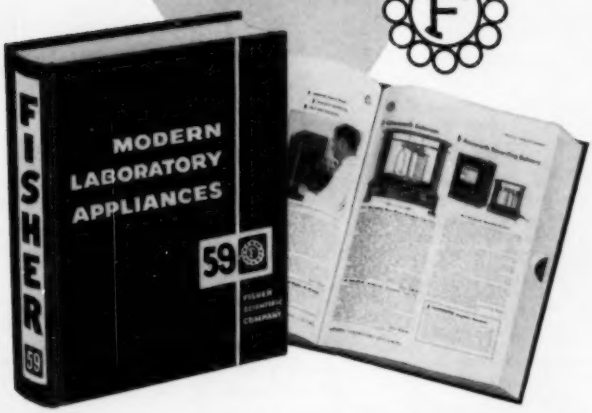

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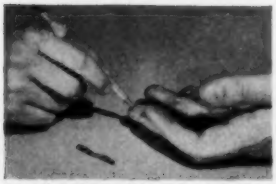

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CLINICAL AND EXPERIMENTAL

EFFECT ON PORTAL BLOOD AMMONIUM OF ADMINISTERING NITROGENOUS SUBSTANCES TO PATIENTS WITH CHRONIC HEPATIC DISEASE

LESLIE T. WEBSTER, JR., M.D.,* CHARLES S. DAVIDSON, M.D., AND GEORGE J. GABUZDA, M.D.

CLEVELAND, OHIO, AND BOSTON, MASS.

IN PATIENTS with cirrhosis and/or Eck's fistula, excessive intakes of protein and certain other nitrogenous substances may precipitate the syndrome of impending hepatic coma† associated with increased blood ammonium concentrations.[1-4] Although the exact nature and sources of increased ammonium in the blood are incompletely understood, it is known that high concentrations of this potentially toxic substance are normally present in portal blood and that these are reduced substantially by the liver.[5-11]

Animal experiments indicate that most of the ammonium in portal blood comes from the large intestine.[6, 12] Moreover, ammonium can be formed in the gastrointestinal tracts of man and experimental animals by means of bacterial enzymatic action on certain nitrogenous substrates and possibly by other means as well.[13-16]

The present investigation of sources contributing to portal blood ammonium in man resulted from the observation that patients with hepatic disease demonstrated variations in blood ammonium concentration during a given day that

From the Department of Medicine, Western Reserve University School of Medicine, at the Cuyahoga County Hospital, Cleveland, Ohio, and from the Thorndike Memorial Laboratory and the Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

This investigation was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract DA-49-007-MD-749.

Preliminary results of this investigation were presented at the sixth annual meeting of the American Association for the study of Liver Disease, Chicago, Illinois, November, 1955. (Abstracts: Am. J. Med. 21: 130, 1956; J. Clin. Invest. 35: 742, 1956.)

Received for publication March 13, 1958.

*Russell M. Wilder Fellow of the National Vitamin Foundation.

†Characterized by mental confusion and a "flapping" type of tremor.

could be largely eliminated by a period of fasting. The feeding of protein produced prompt increases in portal collateral blood ammonium concentration. Some factors responsible for these increases were investigated by determining ammonium concentrations in blood drawn from superficial abdominal veins, peripheral veins, and, in some instances, arteries, both before and after the administration of certain products of protein metabolism, including urea, glutamine, glutamic acid, and ammonium salts. The patients were also studied before and after the oral administration of neomycin sulfate to determine whether or not the increased blood ammonium concentrations resulting from protein and some of its metabolites were dependent upon the integrity of gastrointestinal bacteria.

TABLE I. CLINICAL AND LABORATORY FINDINGS IN 10 PATIENTS WITH HEPATIC DISEASE

PATIENT	AGE AND SEX	DIAGNOSIS*	PHYSICAL FINDINGS†	SERUM TOTAL BILIRUBIN (MG./100 ML.)	SERUM BSP (PER CENT)	BLOOD NONPROTEIN‡ OR UREA NITROGEN (MG./100 ML.)
J. A.	66 M.	Cirrhosis	Icterus, edema	2.6	48	22‡
J. McC.	56 M.	Cirrhosis, pertioneal tuberculosis	Angiomata	1.0	19	12
R. K.	49 M.	Cirrhosis, peptic ulcer	Icterus, angiomata, splenomegaly, edema	4.0	29	26‡
E. G.	76 F.	Cirrhosis	Splenomegaly, edema	0.8	30	17
B. B.	67 M.	Cirrhosis, metastatic carcinoma	Edema	0.9	27	15
A. D.	68 M.	Cirrhosis	Angiomata, edema	1.3	20	17
A. I.	58 M.	Cirrhosis	Angiomata, splenomegaly	2.0	38	13
A. H.	70 F.	Metastatic carcinoma	Edema	0.4	--	17
J. K.	49 M.	Cirrhosis	Icterus, angiomata, edema	3.0	53	12
E. E.	68 M.	Cirrhosis	Edema	1.3	58	14
A. S.	62 M.	Cirrhosis	Edema	1.6	19	17

*The diagnosis was confirmed by histologic examination in all patients except E. G.

†All patients had ascites and dilated superficial abdominal collateral veins. All patients had hepatomegaly except J. McC. and E. G.

‡Blood nonprotein nitrogen.

METHODS

Nine men and two women were studied (Table I). Their ages ranged from 49 to 76 years. All had histories of heavy alcoholic and poor dietary intakes and had physical and laboratory findings consistent with chronic hepatic disease. Diagnoses included Laennec's cirrhosis in 9 patients, carcinoma metastatic to the liver in one patient, and cirrhosis and metastatic carcinoma involving the liver in the remaining patient. Diagnoses were confirmed by histologic findings in all except one patient (E. G.).

Five patients were observed on the ward of the Thorndike Memorial Laboratory at Boston City Hospital, 5 on the medical wards of the Cleveland City Hospital, and 1 on a medical ward at the Crile Veterans Administration Hospital in Cleveland. By calculation, their daily diets contained adequate calories: 60 to 90 Gm. of protein and 250 mg. of sodium. Food intakes were relatively constant and no dietary alterations were made during the course of this study. Except for J. A. (Fig. 1), none of these patients received foods or medications containing nitrogen for at least 12 hours before the initial blood ammonium determinations were performed. None of these patients had confusion, coma, tremor, shock, hemorrhage, or blood urea nitrogen concentrations over 20 mg. per 100 ml.

Ammonium nitrogen (NH_4-N)* was determined in serial blood samples obtained approximately simultaneously from a superficial abdominal vein, from an antecubital vein, and, in some instances, from an artery usually twice before, and at intervals after, the administration of various nitrogenous substances. These observations were repeated in some patients after they had been treated with oral neomycin sulfate.† This essentially nonabsorbed antibiotic was given as follows: 2 Gm. initially, then 1 to 2 Gm. every 4 to 6 hours until the dose totaled 6 to 12 Gm. during the 16 to 20 hours preceding the absorption studies.

Blood samples were obtained from superficial abdominal veins located centrally and superiorly on the abdomens of the patients studied. Employing such abdominal veins as a method for sampling of portal venous blood[17] presented several limitations. Although portal blood NH_4-N levels in human beings are reported to be 2 to 4 times higher than the antecubital venous concentrations,[9] the fasting portal collateral blood NH_4-N concentrations obtained in the present study usually were not much higher than, and sometimes were equal to, those found in antecubital venous blood. The extent to which blood in a given superficial abdominal vein reflected an assumed change in portal blood NH_4-N concentration varied in several ways. Significant differences in fasting abdominal venous blood NH_4-N concentrations and in the increases in blood NH_4-N concentration occurring after a given ammonium load were noted in different patients, in various abdominal veins of the same patient, in different locations of the same abdominal vein, and in the same location on different days. Indeed, following the oral administration of 50 mg. or more of ammonium salt nitrogen, the blood NH_4-N concentration increased in some veins on the anterior abdominal wall while it did not in others indicating that the latter veins did not contain a measurable quantity of portal venous blood. Thus, there were anticipated difficulties in properly assessing the fluctuating blood NH_4-N concentrations obtained and in determining the source of blood obtained from the superficial abdominal veins of patients with ascites and chronic hepatic disease.

Most limitations in assessing fluctuating blood NH_4-N concentrations were overcome by comparing NH_4-N concentrations only in blood samples obtained for each patient from the same location in the same abdominal vein on a given day. Blood NH_4-N concentrations obtained in this way from a fasting patient were quite reproducible (Table II). The same location was also employed when changes in NH_4-N concentration were compared on different days before and after neomycin. When changes in blood NH_4-N concentrations following the administration of a nitrogenous substance were compared before and after neomycin was given, only gross changes were considered significant because of the day-to-day variations in fasting blood NH_4-N concentrations.

Blood obtained from the superficial abdominal veins employed in this study was assumed to represent portal blood to some extent for the following reasons. A superficial abdominal vein was selected so that its blood yielded increases in NH_4-N concentration after the administration of test nitrogenous substances. These increases almost always exceeded or at least equaled those found for antecubital venous blood. If the NH_4-N

*Whether ammonium normally is present in circulating blood or whether it is formed only after blood is shed has not been established. Volatile base, released from blood upon addition of alkali, is assumed to be nearly all ammonium.

†Mycifradin sulfate, The Upjohn Company, Kalamazoo, Mich.

concentration in blood from a superficial abdominal vein did not change significantly after administration of a test nitrogenous substance, the result was included only when a significant increase followed the subsequent administration of quantities of another nitrogenous substance shown by previous experience to produce such an effect (e.g., 60 mg. or more of NH_4-N given orally as an ammonium salt). In 3 patients included in this study (A. I., J. K., and A. S.) and in 3 additional patients given methionine orally,[18] increases in superficial abdominal blood NH_4-N concentration after test nitrogenous substances exceeded those found in arterial blood. Indeed, NH_4-N concentration was frequently significantly increased in superficial abdominal venous blood when significant increases were

TABLE II. DIFFERENCES IN NH_4-N CONCENTRATIONS IN CONSECUTIVE BLOOD SAMPLES DRAWN DURING THE SAME DAY FROM THE SAME SITE IN THE SAME FASTING PATIENT

SITE	DIAGNOSIS	NUMBER OF PATIENTS	NUMBER OF COMPARISONS IN PATIENTS	RANGE NH_4-N DIFFERENCES (μG/100 ML.)
Arm or leg vein	No liver disease	4	6	0-11
	Cirrhosis	19	84	0-23
Artery	No liver disease	7	7	1-12
		11	17	0-18
Superior jugular bulb	No liver disease	7	7	1-15
	Cirrhosis	9	13	1-14
Abdominal collateral vein	Cirrhosis	11	34	0-22

TABLE III. NITROGEN AND AMMONIUM CONTENTS OF SUBSTANCES ADMINISTERED

SUBSTANCE, QUANTITY, AND COMPOSITION	ROUTE	NITROGEN* ADMINISTERED (GM.)	AVERAGE ANALYZED NH_4-N (MG./GM. N)	TOTAL ANALYZED NH_4-N ADMINISTERED (MG.)
Protein, 50 Gm.; Lonalac,† 120 Gm.; 3 eggs and water to 500 ml.	P.O.	8	1.11 (3‡)	8.9
Protein, 30 Gm.; 300 ml. of above mixture	P.O.	4.8	--	--
Protein, 20 Gm.; Lonalac,† 52 Gm.; 1 egg and water to 500 ml.	P.O.	3.2	--	--
L-Glutamine,§ 4 to 10 Gm. given in 250 ml. water	P.O.	0.77-1.9	8.89 (5‡)	6.76-16.9
L-Glutamic acid,¶ 10 Gm. given in 250 ml. water	P.O.	0.76	0.14 (2‡)	0.11
Urea, 0.0017-0.011-0.150 Gm. per minute given in water or in 5 per cent glucose and water	I.V‖	0.0008-0.005-0.070 per minute	1.35 (2‡)	0.0011-0.0068-0.0945 per minute

*The nitrogen contents of Lonalac, L-glutamine, L-glutamic acid, and urea were calculated from data provided by the manufacturer. Each egg was estimated to contain 6 Gm. of protein.

†A milk substitute restricted in sodium content and provided through the courtesy of Mead Johnson & Company, Evansville, Ind.

‡Number of analyses.

§Furnished through the courtesy of Merck & Company, Inc., Rahway, N. J., and Gray Pharmaceutical Company, Inc., Newton, Mass.

¶Given as "Glutavene," a sodium salt of L-glutamic acid, provided through the courtesy of Gray Pharmaceutical Company, Inc.

‖Constant rate infusion.

not detected in arterial or antecubital venous blood. Such would be expected if the changes in blood from the superficial abdominal veins did represent those in portal blood to some extent. While the above observations do not prove conclusively that the superficial abdominal veins selected carried measurable quantities of portal blood, they are at least consistent with such a hypothesis. Subsequently, in this communication superficial abdominal veins will be called portal collateral veins and superficial abdominal venous blood will be referred to as portal collateral blood.

Data concerning the type, composition, quantity, route of administration, nitrogen content, and NH_4-N analysis of each nitrogenous substance employed are presented in Table III.

A modification of the Conway technique was employed to determine ammonium nitrogen in the blood and in the nitrogenous substances administered.[19] Duplicate or triplicate aliquots of blood were injected promptly into the Conway units, and diffusion times of 10 or 16 and ⅔ minutes were employed. Results for blood and all nitrogenous solutions used were calculated as recommended for blood by Conway. The per cent recovery of volatile base from blood was estimated from the per cent ammonium recovered from an ammonium sulfate standard. A modification was introduced at Cleveland, i.e., grease was applied to the rim of the Conway dish rather than to the entire underside of the cover glass. The method as employed in Boston and Cleveland yielded comparable ranges of normal values and of reproducibility.

Serum bilirubin, nonprotein nitrogen, and blood urea nitrogen concentrations were determined by standard techniques.[20-22] Bromsulphalein was determined colorimetrically in serum obtained 45 minutes after the injection of 5 mg. of dye per kilogram of body weight.[23]

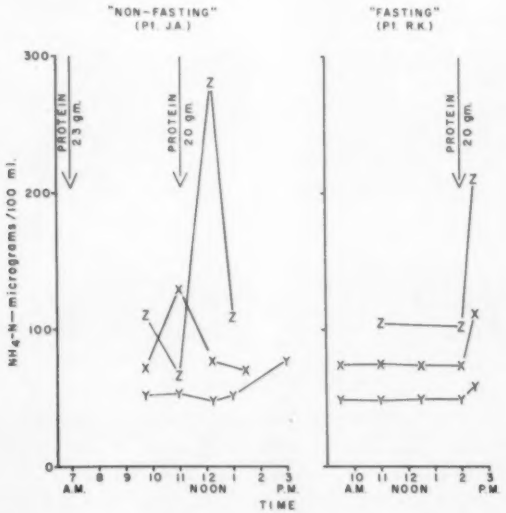

Fig. 1.—"Fasting" and "nonfasting" blood ammonium concentrations in cirrhosis: jugular vein (X); antecubital vein (Y); abdominal collateral vein (Z). A 20 Gm. protein meal provides 3.2 Gm. nitrogen.

RESULTS

Effect of Fasting.—Large variations in NH_4-N concentrations in blood drawn from different sites could occur in nonfasting patients with cirrhosis (J. A., Fig. 1). However, blood NH_4-N concentrations were quite reproducible

in patients with cirrhosis who had not received foods or medications containing nitrogen for at least 12 hours prior to blood sampling (R. K., Fig. 1). Differences between concentrations in two samples of blood drawn at different times during a day from the same site in the same fasting patient ranged from 0 to ±23 μg per 100 ml. (mean, −2; S.D., ±8.1). The differences in patients without liver disease were comparable to those found in alert patients with cirrhosis (Table II).

Fluctuations in blood NH_4-N concentrations with administration of a test nitrogenous substance were considered significant only if they exceeded the range of fluctuation observed for fasting patients (±23 μg per 100 ml.).

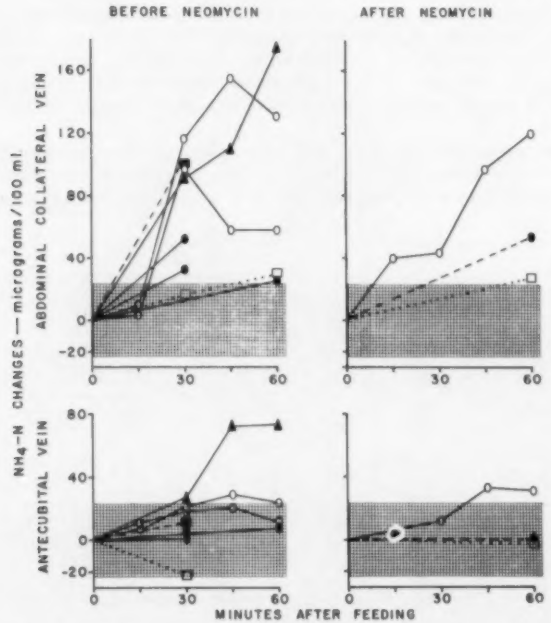

Fig. 2.—Blood ammonium changes with protein feeding before and after neomycin. The symbols represent individual patients: B. B. (▭); R. K. (▬); J. McC. (0); J. A. (●); E. G. (▲). The lines joining the symbols indicate the grams of protein nitrogen ingested: solid line (8 Gm.); dotted line (4.8 Gm.); broken line (3.2 Gm.). The shaded area represents the range of blood NH_4-N concentration reproducibility (Table II).

Effect of Protein Ingestion.—Meals containing from 20 to 50 Gm. of protein were given to 5 patients on 8 occasions. Within an hour these meals consistently produced increased portal collateral blood NH_4-N concentrations. Antecubital venous concentrations were not always similarly affected (Fig. 2). Thirty minutes after ingesting a 50 Gm. protein meal, another patient (A. S., Table IV) had an increase in arterial blood NH_4-N concentration of 57 μg (from 65 to 122 μg) per 100 ml. without a significant change in antecubital venous blood NH_4-N concentrations.

The oral administration of neomycin to 3 of the above 5 patients did not appreciably alter the increases in abdominal collateral blood NH_4-N noted in response to protein feeding. However, neomycin did change the bacterial flora and NH_4-N concentrations in aspirates of mixed gastric and duodenal juice obtained from 2 of these 5 patients (J. McC. and J. A.). The flora changed from one consisting of mixed gram-positive and gram-negative bacteria to one of Monilia. Before neomycin administration, the NH_4-N analyzed in samples of mixed gastric and duodenal juice obtained from one of these fasting patients (J. McC.) on 5 separate mornings ranged from 7.1 to 14.7 mg. per 100 ml. with a mean of 11.6 mg. per 100 ml. After neomycin (7 Gm. over a period of 17 hours) the NH_4-N concentrations in two gastric aspirates were 4.3 and 3.9 mg., respectively, per 100 ml.

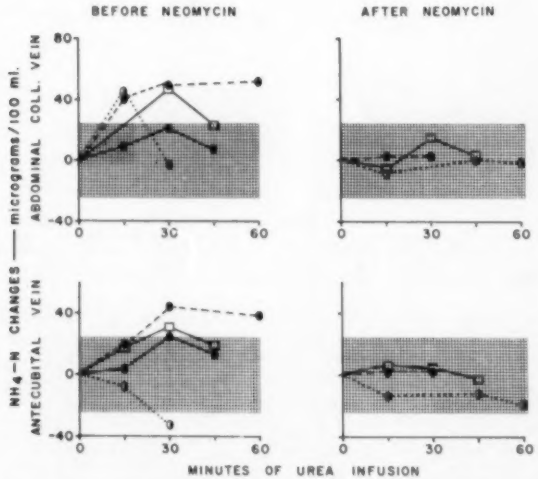

Fig. 3.—Blood ammonium changes during urea infusions before and after neomycin. The symbols represent individual patients: B. B. (□); J. A. (●); E. E. (◑). The lines joining the symbols indicate the grams of urea nitrogen given per minute: solid line (0.0008 Gm.); dotted line (0.005 Gm.); broken line (0.07 Gm.). The shaded area represents the range of blood NH_4-N concentration reproducibility (Table II).

Effect of Urea Infused Intravenously.—Constant rate intravenous infusions providing from 0.8 to 70 mg. of urea nitrogen per minute were given to 3 patients on 4 occasions. Within 15 to 30 minutes, these infusions produced significant rises in portal collateral and antecubital venous blood NH_4-N concentrations in 3 out of 4 instances (Fig. 3). Infusion of 1 mg. urea nitrogen per minute to another patient (A. S., Table IV) resulted in an arterial blood NH_4-N rise of 10 μg per 100 ml. within 30 minutes and in a significant rise of 57 μg per 100 ml. above the control level within 60 minutes of the time the infusion was started.

Prior administration of neomycin prevented significant increases in NH_4-N concentration in blood obtained from both antecubital and portal collateral veins in the same 3 patients. The lack of a portal blood NH_4-N response to infused urea probably was not attributable to a direct inhibitory effect of neomycin

on bacterial urease. Neomycin in concentrations up to 100 μg per milliliter did not inhibit the action of jack bean meal urease in vitro (Table V).

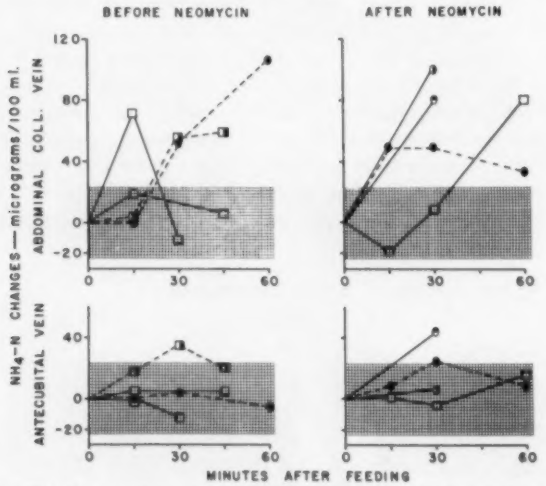

Fig. 4.—Blood ammonium changes with L-glutamine feeding before and after neomycin. The symbols represent individual patients: B. B. (▭); J. A. (●); E. E. (◐); A. I. (◑); A. D. (■). The lines joining the symbols indicate the grams of L-glutamine nitrogen ingested: solid line, 0.77 Gm.; broken line, 1.9 Gm. The shaded area represents the range of blood NH_4-N concentration reproducibility (Table II).

TABLE IV. BLOOD NH_4-N FOLLOWING ORAL GLUTAMINE, PROTEIN, AND INTRAVENOUS UREA

PATIENT	SUBSTANCE	SITE	BLOOD NH_4-N (μG./100 ML.) CONTROL	20 MIN.	30 MIN.	60 MIN.
A. I.*	Glutamine	Antecubital vein	48		92	
	4.0 Gm., P.O.	Artery	52		97	
		Abdominal collateral vein	59		139	
J. K.	Glutamine	Antecubital vein	124	124	141	
	4.0 Gm., P.O.	Artery	115	120	148	
	Protein (casein)	Antecubital vein	58		66	
	50 Gm., P.O.	Artery	65		122	
A. S.	Urea	Antecubital vein	35		40	40
	1 mg. urea N per min. I.V.†	Artery	53		63	110

*Received 12 Gm. neomycin in preceding 18 hours.
†Infusion continued for 60 minutes during which time values were obtained.

TABLE V. IN VITRO EFFECT OF NEOMYCIN ON NH_4-N PRODUCTION BY UREASE

UREA-N (μG)	UREASE (μG)	NEOMYCIN (μG)	NH_4-N PRODUCED (μG/ML.) NO. 1	NO. 2	AVERAGE
20	100	0	17.3	17.7	17.5
20	100	100	17.3	18.2	17.8
40	100	0	34.0	32.9	33.5
40	100	100	35.8	36.5	36.2
20	150	0	19.4	19.3	19.4
20	150	100	18.3	18.8	18.6
40	150	0	37.2	38.1	37.7
40	150	100	36.7	37.9	37.3

Effect of L-*Glutamine Ingestion.*—Within an hour of the ingestion of L-glutamine (4 to 10 Gm.) 3 patients showed significant increases in portal collateral blood NH_4-N concentrations in 3 out of 4 instances and in antecubital venous blood NH_4-N concentration in one instance (Fig. 4). Patient A. I. (Table IV) with an increase in portal collateral NH_4-N concentration of 80 μg per 100 ml. had a simultaneous increase in the arterial level of 45 μg per 100 ml. In addition, Patient J. K. (Table IV) sustained an increase of 33 μg per 100 ml. in arterial blood NH_4-N concentration 30 minutes after he ingested 4 Gm. of L-glutamine.

The only patient (B. B.) in whom a significant rise in portal collateral blood NH_4-N was not noted had received 10 Gm. of L-glutamic acid orally one hour previous to the administration of L-glutamine. Later, when this patient was given L-glutamine without prior glutamic acid, a significant portal collateral NH_4-N increase occurred.

Increases in portal collateral blood NH_4-N concentrations which followed the ingestion of L-glutamine occurred despite neomycin administration in 2 patients (J. A. and B. B., Fig. 4) previously tested without this antibiotic, and in 2 additional patients (A. I. and E. E., Fig. 4). Increases did not occur in 3 of these neomycin-treated patients after urea was given intravenously (J. A. and E. E.) or methionine was given orally (A. I.) about one hour prior to glutamine administration. Thus, neomycin given orally prevented portal blood NH_4-N increases which followed urea and methionine administration but did not prevent those which resulted from the ingestion of glutamine.

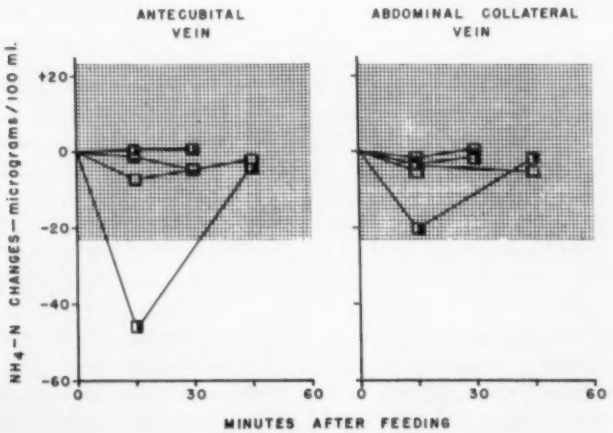

Fig. 5.—Blood ammonium changes with L-glutamic acid feeding. The symbols represent individual patients: B. B. (□); A. D. (■); A. H. (▬). The quantity of L-glutamic acid nitrogen ingested was 0.76 Gm. The shaded area represents the range of blood NH_4-N reproducibility (Table II).

Effect of L-*Glutamic Acid Ingestion.*—Ten grams of L-glutamic acid, given orally as sodium glutamate, produced either no change or decreases in portal

collateral and antecubital venous blood NH_4-N concentrations in 3 patients on 4 occasions (Fig. 5). The effect of this amino acid was not studied after the administration of neomycin.

Effect of Ammonium Salt Ingestion.—The maximum amount of volatile base found by analysis in any of the above nitrogenous substances administered orally was equivalent to approximately 17 mg. of NH_4-N (Table III). Accordingly, quantities of ammonium salts containing more than this amount of nitrogen were administered orally to 3 of the patients studied. Significant rises in portal collateral or antecubital venous blood NH_4-N concentrations did not occur when 20 to 25 mg. of NH_4-N was given orally to 3 patients on 4 occasions. Significant rises were noted in portal collateral but not in antecubital venous blood NH_4-N concentrations when 50 mg. of NH_4-N was given to one and when 60 or 370 mg. was given to another of these patients (Table VI).

TABLE VI. THE EFFECT OF INGESTED AMMONIUM SALTS* ON BLOOD NH_4-N CONCENTRATIONS

			BLOOD NH_4-N CONCENTRATION (μG PER 100 ML.)									
			ANTECUBITAL VEIN (MINUTES)					ABDOMINAL COLLATERAL VEIN (MINUTES)				
PATIENT	SALT	NH_4-N (MG.)	0	15	30	45	60	0	15	30	45	60
J. A.	NH_4Cl	20	50	50	49	49		60	60	60	60	
	NH_4 Citrate	60	52	52	59	50	50	103	138	170	130	101
	NH_4 Citrate	370	57		57			52		133		
B. B.	NH_4Cl	25	76	79	79	82		108	110	106	102	
	NH_4Cl	25	99	94	99	103		107	105	117	104	
A. D.	NH_4Cl	22	36	35	38			83	77	81		
	NH_4Cl	50	37	46				44	72			

*Given in 240 ml. of water.

DISCUSSION

Although the studies of Folin and Denis[6] indicate that the large intestine is the chief and most constant source of portal blood ammonia in animals, the time interval in which increases in the portal collateral blood NH_4-N concentration were noted in the patients with chronic hepatic disease reported here suggests that the upper gastrointestinal tract may be an important site of ammonium absorption shortly after protein ingestion in man (Fig. 2). Subsequent studies were directed toward elucidating some of the factors responsible for the prompt increases in portal collateral blood NH_4-N concentration occurring after protein ingestion.

In animals, bacterial urease is responsible for ammonium production from urea which has entered the gastrointestinal tract from circulating blood.[13] The experiments of Dintzis and Hastings in mice showed that intestinal ammonium production from urea was largely abolished by the administration of mixed penicillin, sulfaguanidine, and oxytetracycline. These investigators concluded that the antibiotic mixture eliminated urease activity predominantly by effecting a reduction in bacterial population. Neither the antibiotic mixture, a homogenate of gastrointestinal tissues from antibiotic-fed mice, nor a fecal homogenate from an antibiotic-fed mouse was found to inhibit the activity of urease.

The increased portal collateral and arterial blood NH_4-N concentrations noted during intravenous infusions of urea probably resulted from a breakdown of circulating blood urea in the gastrointestinal tracts of the patients with chronic hepatic disease. The breakdown appeared dependent on gastrointestinal bacterial action because increased portal collateral blood NH_4-N concentrations were not observed during urea infusions given the same patients who had been previously treated with neomycin. Neomycin sulfate, selected because it is bacteriocidal and poorly absorbed from the gastrointestinal tract,[24] altered the gastric flora and caused decreased NH_4-N concentrations of mixed gastric and duodenal juice in patients with chronic hepatic disease, but did not inhibit the action of jack bean meal urease in vitro.

It appeared unlikely that urea produced from ingested protein, and subsequently broken down in the gut, was solely responsible for the portal collateral blood NH_4-N increases which took place after protein ingestion because these increases, unlike those occurring during urea infusions, were not modified appreciably in patients treated with neomycin. Increases in abdominal collateral blood NH_4-N concentrations following the ingestion of glutamine likewise were not significantly modified by the prior oral administration of neomycin sulfate in patients in whom this antibiotic had modified the prompt response to intravenous urea or oral methionine (Fig. 4). Thus, protein and one of its constituents, glutamine, produced prompt portal collateral blood NH_4-N increases which were probably independent of bacterial action. The amide rather than the amino nitrogen of glutamine seemed responsible for these increases because glutamic acid, which is similar to glutamine except that it lacks an amide nitrogen, did not produce rises in portal collateral blood NH_4-N concentrations (Fig. 5). The fact that the amide-nitrogen of glutamide and of γ-glutamine peptides is easily hydrolyzed[25-27] supports the above hypothesis.

The results obtained with glutamine must be applied with caution to protein for several reasons. First, glutamine was not administered with other amino acids which result from protein hydrolysis. Some of these other amino acids possibly might modify its effect on portal blood NH_4-N concentrations. Second, the amount of glutamine administered (4 to 10 Gm.) may have exceeded that present in the quantities of protein given (20 to 50 Gm.). From available data it is difficult to determine the glutamine content of proteins because, after its amide group is hydrolyzed, glutamine is analyzed as glutamic acid. It is not known whether or not a smaller quantity of glutamine than given here would have produced a portal blood NH_4-N increase. Third, the similarity of the blood NH_4-N responses to glutamine and protein does not mean that glutamine is the only protein constituent which might produce a portal blood NH_4-N increase independently of bacterial action. In this regard, and contrary to previously reported results,[28-30] methionine given orally may produce a portal blood NH_4-N increase which is largely abolished by prior administration of neomycin.[18] The role of other amino acids and protein constituents in gastrointestinal NH_4-N production in man remains to be evaluated.

The possibility was considered that free NH_4-N contained in protein or glutamine could account for the blood NH_4-N increases obtained with these orally

administered substances. However, ingestion of ammonium salts containing more NH_4-N (20 to 25 mg.) than found by analysis of protein and glutamine produced little effect on portal collateral blood NH_4-N concentrations (Table IV). Urea given intravenously did not produce increases in blood NH_4-N concentrations in patients treated with neomycin. This indicates that the increases noted with urea given prior to neomycin administration could not be attributed to free NH_4-N contained in this compound (Fig. 3).

Twenty to twenty-five milligrams of NH_4-N administered orally as an ammonium salt failed to produce increases in portal collateral blood NH_4-N concentration whereas similar quantities of nitrogen given as urea intravenously did result in significant increases. This apparent inconsistency is unexplained.

Investigating the sources of ammonium derived from ingested protein may have application in the treatment of the hepatic coma syndrome. Results of this study only suggest that ammonium production from glutamine is a potential cause of protein-induced hepatic coma. Protein usually must be fed over longer periods than covered by these observations in order to induce the syndrome of impending hepatic coma in susceptible patients. Furthermore, antecubital venous blood NH_4-N concentrations usually did not increase with single doses of protein or glutamine to levels often observed in impending hepatic coma. However, determinations of arterial blood NH_4-N concentrations after administration of both these substances to 2 patients with severe hepatic disease did yield some concentrations in the "coma range."

That ammonium production by gastrointestinal bacteria may be important in the genesis and outcome of hepatic coma is suggested by the favorable clinical results obtained with neomycin.[31] The observation that neomycin orally caused a decrease in gastrointestinal ammonium production from urea given intravenously provides further rationale for its therapeutic use in this condition (Fig. 3). The use of neomycin might be particularly applicable in the management of patients with hepatic coma complicated by azotemia, since alert patients with cirrhosis and azotemia may display unusually elevated fasting arterial blood NH_4-N levels.[32] However, the failure of neomycin to eradicate the portal collateral blood NH_4-N increases occurring shortly after protein ingestion (Fig. 2) suggests that treatment with this antibiotic should not replace an appropriate degree of protein restriction in the regimen for the management of patients with hepatic coma.

SUMMARY

The effect of administering several nitrogenous substances upon concentrations of ammonium in superficial abdominal (portal collateral) venous blood was studied in patients with chronic hepatic disease. To evaluate the influence of bacteria on ammonium production in the gastrointestinal tract, some of the patients were similarly studied after neomycin sulfate was given orally.

Prompt increases in portal collateral blood ammonium nitrogen concentrations followed the administration of 20 to 50 Gm. of protein orally, of 4 to 10 Gm. of glutamine orally, of 0.8 to 70 mg. of urea nitrogen per minute intravenously, and of 50 mg. or more of ammonium salt nitrogen orally. Similar

increases did not result from the ingestion of 10 Gm. of L-glutamic acid or 25 mg. or less of nitrogen given orally as an ammonium salt. The quantity of ammonium nitrogen found by analysis of the protein, glutamine, and urea employed in these studies was not great enough to explain the increases in portal collateral blood ammonium nitrogen concentrations resulting from the administration of these substances.

Portal collateral blood NH_4-N increases that occurred during intravenous infusions of urea were depressed by oral neomycin sulfate, probably indicating that the infused urea entered the gastrointestinal tract and that ammonium production from this urea was dependent upon the action of gastrointestinal bacteria. Protein and glutamine were similar in that the prompt portal blood ammonium nitrogen increases noted shortly following their oral administration were not affected significantly by oral neomycin and, therefore, apparently were not dependent on bacterial action.

The data suggest that ammonium (or a related substance) is absorbed from the upper gastrointestinal tract shortly following the ingestion of protein and of a protein constituent, L-glutamine. Amide rather than amino nitrogen was probably responsible for the increases in concentration of ammonium nitrogen in portal collateral blood resulting from the ingestion of glutamine, because glutamic acid given orally did not produce this effect.

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A SIMPLE NEW METHOD FOR ESTIMATING CARDIAC OUTPUT

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ALTHOUGH reliable techniques are available for measuring cardiac output in man, they are too complex for widespread clinical use. While studying the relation of blood flow and Rb^{86} uptake in the tissues of dogs, it was found that the cardiac output could be estimated by merely measuring the Rb^{86} concentration reached in the arterial plasma during a standard intravenous infusion of isotope. An infusion pump and counting equipment are the only necessary apparatus. It should be possible to determine cardiac output by this method in man by obtaining a single specimen of arterial blood after administering less than 10 μc of Rb^{86} over a 5-minute period.

MATERIALS AND METHODS

Multiple venous catheters were placed in 14 mongrel dogs (8 to 17 kilograms) anesthetized with pentobarbital. In all animals Rb^{86} was injected at the same continuously decreasing rate[1] for 6 to 24 minutes through a cardiac catheter in the pulmonary artery. Specimens of mixed venous blood were obtained from the pulmonary artery proximal to the site of injection of Rb^{86}. Cardiac output was measured using the Fick principle, making calculations from the total amount of Rb^{86} taken up in the tissues perfused by the systemic circulation and the mean arteriovenous difference in plasma Rb^{86} concentration. Thus,

$$\text{cardiac output} = \frac{Rb^{86}\text{ uptake}}{\text{mean arteriovenous difference in plasma } Rb^{86}\text{ concentration}}$$

where: Rb^{86} uptake = Rb^{86} injected − Rb^{86} in blood − Rb^{86} in lungs.

If it is assumed that the blood is one-fourth arterial and three-fourths venous, and totals 10 per cent of the body weight,[2] then at the end of the infusion of isotope:

Rb^{86} in blood = 0.1 Kg. body wt. (0.25 Rb^{86} c.p.m./ml. arterial blood + 0.75 Rb^{86} c.p.m./ml. mixed venous blood).

All lung tissue was digested in HNO_3 for determination of Rb^{86} content.[3]

In 5 additional dogs cardiac output was measured by the dye dilution principle of Stewart and Hamilton[4, 5] employing T-1824. These determinations were made during the last minute of measurement of cardiac output by Rb^{86} infusion.

The catheters were also placed in the superior vena cava, the inferior vena cava above the liver, an hepatic vein, a renal vein, and the inferior vena cava distal to the kidneys. Several blood specimens were obtained from each vein during the infusion of Rb^{86}. The total Rb^{86} content of each lung, the heart, the kidneys, and the organs of the portal bed (liver, stomach, intestines, spleen, and pancreas) was determined by digestion of the tissue.

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One of the dogs was studied with an open chest employing a respirator. In 4 additional animals cardiac output determinations only were carried out. These dogs were bled 200 ml. prior to study and received l-norepinephrine by infusion as a part of a simultaneous study of coronary blood flow.

The total blood flow to the portal bed and the kidneys was obtained from their Rb^{86} content and the mean arteriovenous Rb^{86} difference during the infusion. It has been found previously that the brain takes up only 0.1 to 0.2 per cent of injected Rb^{86} up to 30 minutes after the start of the infusion[3]; therefore, the Rb^{86} content of the brain was disregarded in making calculations. It was assumed that the heart extracted 70 per cent of the isotope in coronary plasma.[1] The Rb^{86} content of the remainder of the body was obtained by difference. The blood obtained from the inferior vena cava below the kidney was assumed to be representative venous sample for this tissue, composed chiefly of muscle, skin, and bone. To calculate the extraction of Rb^{86} by the lungs, the blood flow to each lung was assumed to be one-half of the total cardiac output.

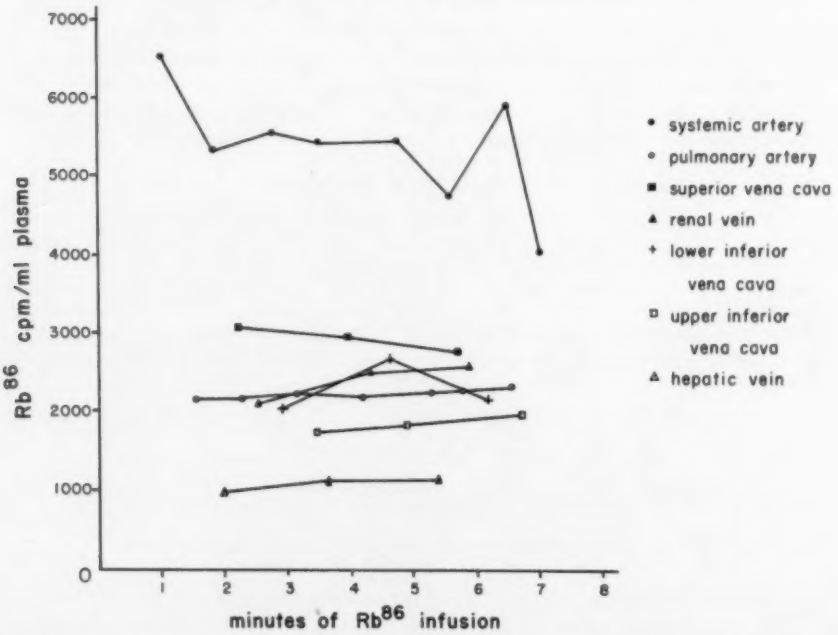

Fig. 1.—Radioactivity of plasma from several sites during an intravenous infusion of Rb^{86}.

RESULTS AND COMMENT

Illustrative data for one dog are shown in Fig. 1.

Cardiac Output.—The mean Rb^{86} concentration in the arterial plasma of each dog, when corrected for variation in the amount of Rb^{86} injected, was closely correlated with the calculated cardiac output. For convenience the radioactivity of arterial plasma in relation to the amount of Rb^{86} injected was plotted as a ratio (Fig. 2). The ordinate and abscissa of Fig. 2 correlate with an r of +0.97. Therefore, the calculated cardiac output could be predicted with a mean

error of less than 10 per cent from the plasma concentration of Rb^{86}, which can be readily measured in man. From the intersection of the regression line with the x axis in Fig. 2, the arterial plasma volume can be calculated. A value of 1.7 per cent of the body weight was obtained, which is a reasonable result.

The possibility that the correlation of calculated cardiac output and plasma Rb^{86} concentration might be "spurious"[6] was considered, because the values for body weight and Rb^{86} concentration of the plasma and the injectant entered in a similar relationship into the calculation of both the ordinate and abscissa. Fig. 3 shows that the cardiac output predicted from the Rb^{86} concentration of the plasma agreed very closely with the value obtained by the entirely separate dye technique. Since cardiac output and plasma radioactivity changed significantly during the period of Rb^{86} infusion in one of the animals, separate estimates of output were made in each dog using the mean plasma Rb^{86} concentration and the concentration present immediately before the dye injection. Errors in the measurement of Rb^{86} concentration of the plasma and the injectant affected the corrected plasma Rb^{86} concentration and the cardiac output value obtained from complete Rb^{86} data in the same direction. Therefore, the total error in estimating true cardiac output from plasma Rb^{86} concentration was the error in predicting the value which would be obtained from calculation of cardiac output from complete Rb^{86} data, plus the amount by which this calculation failed to measure cardiac output accurately. The latter error is not directly measurable. Except for the error in estimation of blood volume, the errors of the Rb^{86} method for measuring cardiac output are the same as those of the standard direct Fick procedure employing right heart catheterization. Approximately 10 per cent of the Rb^{86} injected remained in the blood at the end of the infusion, so that relatively little error in the determination of cardiac output would result from large errors in estimation of blood volume.

Distribution of Plasma Flow.—If blood flow was shunted from an area which extracted most of the Rb^{86} in plasma to one which removed but little, there would be a rise in systemic plasma Rb^{86} concentration, even though total cardiac output did not change. Therefore, it was of interest to estimate the variations in rate of flow to various areas by the methods indicated above, and to measure the regional differences in the degree of extraction of Rb^{86}. The variations noted in the partition of blood flow are indicated in Fig. 4, and the range of the per cent of Rb^{86} extracted is shown in Fig. 5. In the lungs, Rb^{86} extraction varied from 3 to 10 per cent and averaged 7 per cent. Despite the differences in partition of plasma flow and in Rb^{86} extraction, which were partly related to the magnitude of cardiac output, the range of variation in the extraction of Rb^{86} by the systemic circulation was very narrow compared to the differences in cardiac output (Fig. 5). These data establish that shunting of blood flow could cause error in the estimation of cardiac output from Rb^{86} uptake, but there was little error of this type in these dogs, despite a wide range of cardiac output (Fig. 2).

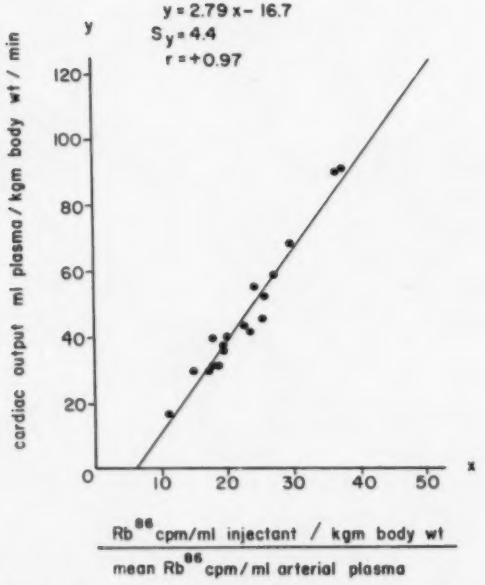

Fig. 2.—Relation of cardiac output calculated from Rb^{86} uptake and the radioactivity of arterial plasma during intravenous infusion of isotope in dogs. Plasma radioactivity is shown as a ratio with the amount of Rb^{86} injected.

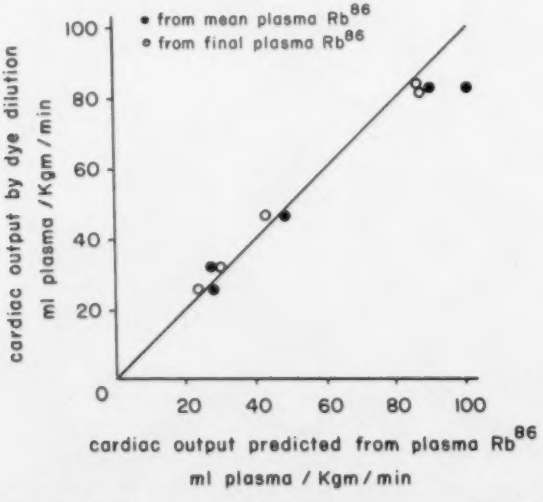

Fig. 3.—Relation of cardiac output obtained by the dye dilution technique to that predicted from the mean plasma Rb^{86} concentration and the plasma Rb^{86} concentration immediately before injection of dye.

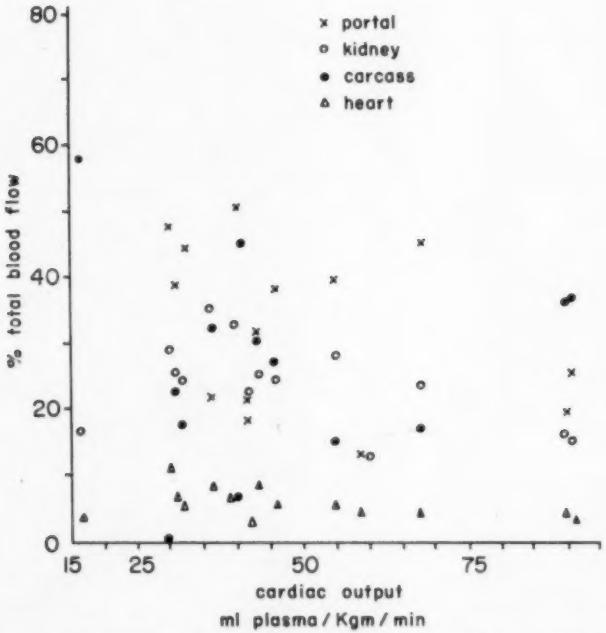

Fig. 4.—Partition of blood flow in dogs at various levels of cardiac output.

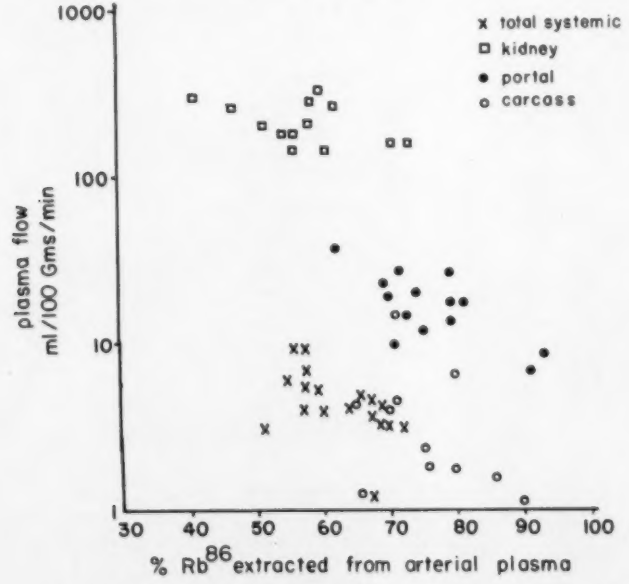

Fig. 5.—Mean plasma flow and Rb^{86} extraction in several areas in dogs.

Results in Man.—A group of patients who were given standard intravenous infusions of Rb^{86} have been previously reported.[7] Applying the values from the regression line in Fig. 2 to these subjects resulted in a mean value for cardiac output of 2.53 L./M.^{2}/min. in 16 patients without heart disease and 1.52 L./M.^{2}/min. in 7 subjects with angina pectoris. Intermediate values were obtained in those with other forms of heart disease.

DISCUSSION

Cardiac output in this group of dogs approximated the normal levels in man, but was lower than that in most other series.[4, 8-10] This was probably largely caused by a long period under pentobarbital anesthesia in the supine position and, in some cases, to significant blood loss and operative trauma. The results of calculations of cardiac output in man from constants derived from studies in dogs indicate only that reasonable values are obtained. The relationship of plasma Rb^{86} concentration and cardiac output in normal man and patients with a variety of diseases must be determined before the Rb^{86} infusion method can be established as a reliable method for clinical use. Blood flowing to the brain or through arteriovenous fistulas would not be measured. It is interesting to speculate that shunting of blood flow to the brain may be in part responsible for high plasma Rb^{86} concentrations in patients with angina pectoris. It is not yet known whether a plasma specimen from a peripheral vein can be substituted for an arterial specimen. Nor have manual methods of injecting the isotope been tested.

In addition to ease of performance and the small amount of equipment necessary, this method has the advantage that changes in cardiac output occurring during a continuing infusion of Rb^{86} could be detected from the resulting change in plasma radioactivity. The blood flow to any tissue in experimental animals can be determined from its Rb^{86} uptake, if representative venous samples can be obtained. Although Rb^{86} is a convenient isotope, it is likely that some other isotopes would also be suitable. It is possible that a chemical substance which is readily measured might be substituted for the tracer.

SUMMARY

1. Fourteen dogs received standard intravenous infusions containing Rb^{86} while serial blood specimens were taken from an artery and multiple veins. Cardiac output was calculated from the amount of isotope taken up by the tissues and the arteriovenous difference in plasma radioactivity.

2. Cardiac output could be predicted with a mean relative error of less than 10 per cent from the Rb^{86} concentration which was reached in the arterial plasma during the infusion.

3. It was possible to measure the average plasma flow to any organ or tissue except the brain by obtaining a representative venous specimen and measuring its total Rb^{86} content at sacrifice.

4. Although the extraction of isotope and the partition of blood flow varied in each tissue and animal, there was only a small difference in the over-all extraction of Rb^{86} by the circulation as a whole, despite a large range of cardiac output.

5. The relationship between plasma Rb^{86} concentration and cardiac output in dogs was applied to the data from a group of human subjects given infusions of isotope previously. The mean value for cardiac output obtained in patients without heart disease was 2.53 L./M.^{2}/min.

6. Since this method is simple and requires but little apparatus, its further calibration in man is indicated.

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INTERRELATIONSHIP BETWEEN SERUM CALCIUM LEVELS: CALCIUM^{45} AND STRONTIUM^{85} METABOLISM IN MAN

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CALCIUM requirements of the human body vary considerably in various physiologic states such as growth, pregnancy, lactation,[1] aging,[2] and in pathologic conditions of the skeleton.[3] Since the dietary calcium intake fluctuates greatly even in the same subject and varies widely in different persons, the problem arises whether a mechanism exists which regulates the intestinal absorption of calcium to meet the different requirements of the human body and thereby maintains calcium homeostasis.

The intestinal absorption of calcium was investigated up to the recent past by metabolic balances of normals and of patients with disturbances of calcium metabolism.[4] Indications were obtained that intestinal absorption of dietary calcium varies in different subjects and seems to depend on the body's needs.[5-8] In recent communications from this laboratory a technique was described employing Ca^{45} which permits the measurements of the utilization of dietary calcium, of the endogenous fecal calcium, and the estimation of digestive juice calcium in man. This technique permits the separation of the fraction of stool calcium which is of endogenous origin from the dietary calcium which passes unabsorbed through the intestinal canal.[9] Indications were obtained that the regulation of absorption of dietary calcium plays a major role in the maintenance of calcium homeostasis.[10]

The metabolism of strontium in man became of considerable interest in recent years since Sr^{90} is one of the important fall-out products of nuclear fission. It was assumed that strontium follows the metabolic pathway of calcium in the human body. The metabolism of radiostrontium was studied in man in this laboratory for the past 3 years and significant differences were noted in the metabolism of calcium and strontium: the intestinal absorption of strontium is less efficient than that of calcium and significantly more of the absorbed strontium than of the absorbed calcium is excreted through the kidneys.[11]

It became of interest to investigate whether acute changes of the level of serum calcium influence the absorption and renal elimination of calcium and strontium. This communication deals with data obtained in two patients who received tracer doses of Ca^{45} and Sr^{85} orally during normocalcemic, hypo-, and hypercalcemic states.

This study was supported in part by a research grant (C-1540) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and by the U. S. Atomic Energy Commission, Contract AT(30-1)-1763.

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MATERIALS AND METHODS

Three studies were performed in each of two patients who received a constant low-calcium diet on the metabolic research ward.

Subject 1 was a 59-year-old white semiambulatory woman in whom the diagnosis of multiple myeloma was established 10 months prior to the study. The patient was in good general condition and received 50 mg. of mytatrienediol (weak estrogen) intramuscularly per day. She had no anemia or albuminuria and the renal functions were normal. Serum calcium was 10.6 mg. per cent, serum inorganic phosphorus 3.0 mg. per cent, and the alkaline phosphatase was 1.7 Bodansky units. Total serum proteins were 8.1 Gm. per cent, albumin 3.2 Gm. per cent, and globulin 4.9 Gm. per cent. Electrophoretic analysis of the serum revealed that the γ-globulin fraction was 30.5 per cent of the total protein.

Subject 2 was a 63-year-old ambulatory white woman in whom the diagnosis of osteoporosis was made 9 years prior to the study. At that time she received a short course of hormone therapy and was not treated thereafter. The patient was in very good physical condition. Serum calcium was 10.3 mg. per cent, inorganic phosphorus 3.8 mg. per cent, and alkaline phosphatase 4.4 Bodansky units.

A solution of calcium^{45} and strontium^{85} containing 25 mg. Ca^{++} carrier was administered as the chloride orally with breakfast on the first day of each study. In the control study, an infusion of 500 ml. of normal saline was started 30 minutes prior to the administration of these tracers and was continued at a constant rate for exactly 6 hours. In the second study, 6 Gm. ethylenediaminetetraacetic acid (Na-EDTA) having a binding capacity of 648 mg. Ca^{++} was infused in saline over a 6-hour period in order to depress the level of ionized serum calcium. In the third study, 69 ml. calcium gluconate containing 648 mg. Ca^{++} was added to the saline infusion in order to raise the serum calcium level. The duration of the studies ranged for 13 to 19 days and the total duration was 64 and 41 days for the two patients, respectively.

Ca^{45} of high specific activity and carrier-free Sr^{85} were used as tracers. The dose of radioactivity was so selected as to permit accurate counting of the biologic samples for a period of approximately 2 weeks with the equipment available in this laboratory. For the evaluation of the subsequent doses, appropriate corrections were made for the radioactivity attributable to the preceding doses.

Blood samples were drawn at 1, 3, 5, 14, and 24 hours after the administration of the tracers. Thereafter, daily blood samples were obtained in the postabsorptive state during the first week and subsequently 3 times weekly in each study. Urine was collected through an indwelling catheter on the first day of each study, the bladder being rinsed with saline at the completion of the urine collections obtained at 2, 4, 6, and 24 hours following the administration of the tracers. On subsequent days, the 24-hour urine output was collected. Stool specimens were collected daily. Calcium, Sr^{85}, and Ca^{45} were determined on each specimen.

Ca^{45} was determined on unashed samples of serum and urine and on acid-ashed samples of stool after oxalate precipitation. The radioactivity was measured in a Q-gas Geiger-Muller counter. Sr^{85} of plasma and urine was measured in a Nuclear scintillation well counter. Since the Sr^{85} radioactivity of the stool specimens was high for several days after the ingestion of the tracer, these samples were counted in a bismuth cathode gamma counter; thereafter, stool aliquots weighing up to 5 Gm. were counted in the scintillation counter.

The details of the radiochemical techniques were previously described.[10, 11] The statistical counting error varied; however, in the initial phases of the experiment sufficient counts were taken to keep the probable error within 2 per cent. In the later phases, counting rates were lower, and it was not feasible to maintain this precision, so that counting errors as high as 5 per cent were incurred. Standards were prepared for each patient at the time of administration of the isotope by delivering the same dose into a volumetric flask; appropriate dilutions were then prepared for counting. The standards were counted along with each set of experimental samples, thereby correcting for the decay of the isotope.

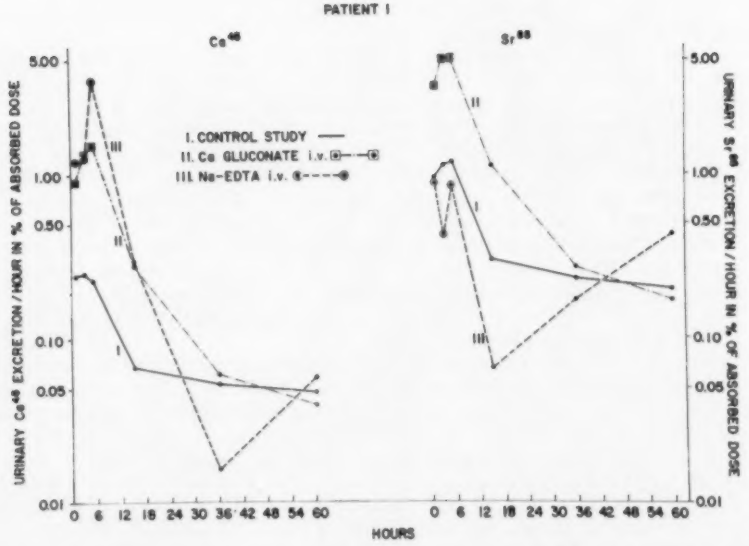

Fig. 1.—Effect of intravenous calcium gluconate and of Na-EDTA on urinary Ca^{45} and Sr^{85} excretion.

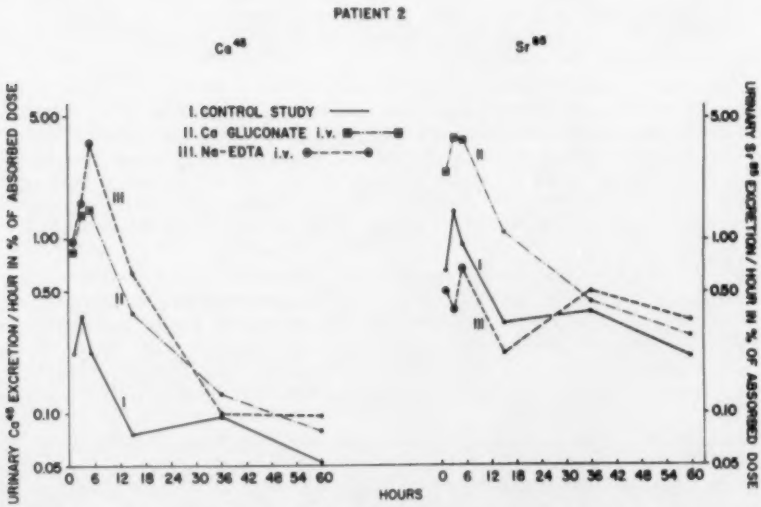

Fig. 2.—Effect of intravenous calcium gluconate and of Na-EDTA on urinary Ca^{45} and Sr^{85} excretion.

Balances of nitrogen, calcium, and phosphorus were measured by analyses of aliquots of 6-day pools of the diet and excreta during the entire period of study. The body weight, fluid balances, urinary creatinine, calcium, and phosphorus were determined daily.

The urinary calcium was determined by the method of Shohl and Pedley,[12] the serum calcium by the Clark and Collip modification of Kramer and Tisdall's method.[13] Dietary and fecal calcium were determined in acid-ashed aliquots using the Clark and Collop technique. The inorganic phosphorus content of serum, urine, and alkaline ash of stool was measured by the method of Fiske and SubbaRow[14]; stool nitrogen was determined by the Kjeldahl method.

RESULTS

I. *Control Study.—*

Metabolic balances: The average values are listed in Table I. The calcium balances of both patients receiving a low-calcium intake were normal.

TABLE I. METABOLIC BALANCES

SUBJECT	TYPE OF STUDY	DURATION OF STUDY DAYS	CALCIUM MG./24 HOURS			
			INTAKE	URINE	STOOL	BALANCE
1	Control	18	153	84	132	-63
	Na-EDTA	19	134	78	114	-58
	Ca-gluconate	14	145	79	145	-79
2	Control	14	180	82	171	-73
	Na-EDTA	14	197	119	181	-103
	Ca-gluconate	13	165	99	178	-112

Calcium^{45} study: The plasma Ca^{45} levels rose gradually, reached a peak of 4.2 and 4.5 per cent, respectively, at the third hour and declined thereafter with time. The daily Ca^{45} excretions are listed in Tables II and III; the rates of urinary Ca^{45} excretions in the first 60 hours of each study are graphed in Figs. 1 and 2. The stool collections of Subjects 1 and 2 contained 50.85 and 63.92 per cent of the administered dose, respectively, in 13 days following the administration of the tracer. The unabsorbed fraction of the dose was estimated by subtracting the endogenous fecal calcium excreted during this period of time from the total stool radioactivity; the absorbed dose of Ca^{45} was 57 per cent for Subject 1 and 42 per cent for Subject 2. The cumulative urinary Ca^{45} excretion in 13 days was 7.27 per cent in Subject 1 and 6.11 per cent in Subject 2, corresponding to approximately 15 per cent of the absorbed Ca^{45}.

Strontium^{85} study: Tables II and III show that the stools of the patients contained 76.55 and 87.01 per cent of the dose in 13 days. It was estimated that 27 per cent of the dose was absorbed by Subject 1 and 15 per cent by Subject 2. The urinary Sr^{85} excretion was 11.10 and 6.85 per cent of the administered dose, or approximately 45 per cent of the *absorbed* dose.

Comparison of Ca^{45} and Sr^{85} metabolism: The absorption of radiostrontium was significantly lower and the urinary excretion of the absorbed strontium significantly higher than that of calcium in both patients. This latter observation is illustrated by the Ca^{45}/Sr^{85} clearance depicted in Fig. 3. The ratio of

Sr^{85}/Ca^{45} in stool to that in plasma was initially much higher, due to the presence of the unabsorbed traces in the stool specimens. Thereafter, these ratios were parallel to each other, the fecal values being slightly above those of the plasma. This is illustrated by the control study of Subject 1 in Fig. 4.

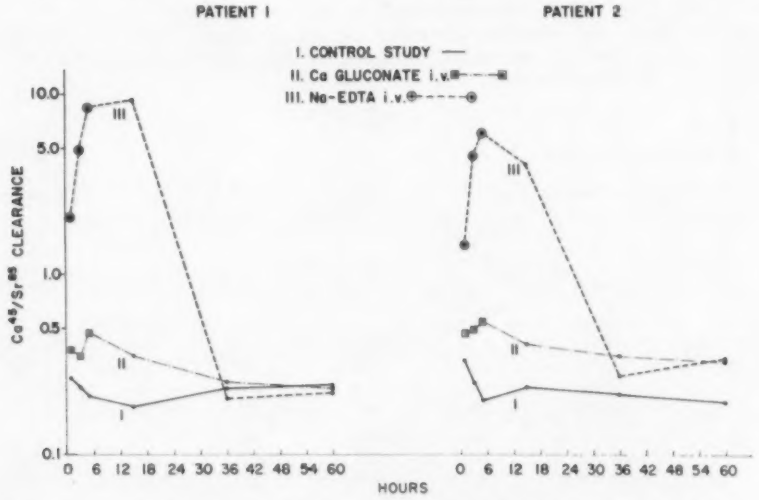

Fig. 3.—Effect of intravenous calcium gluconate and of Na-EDTA on the ratio of clearance of Ca^{45}/Sr^{85}.

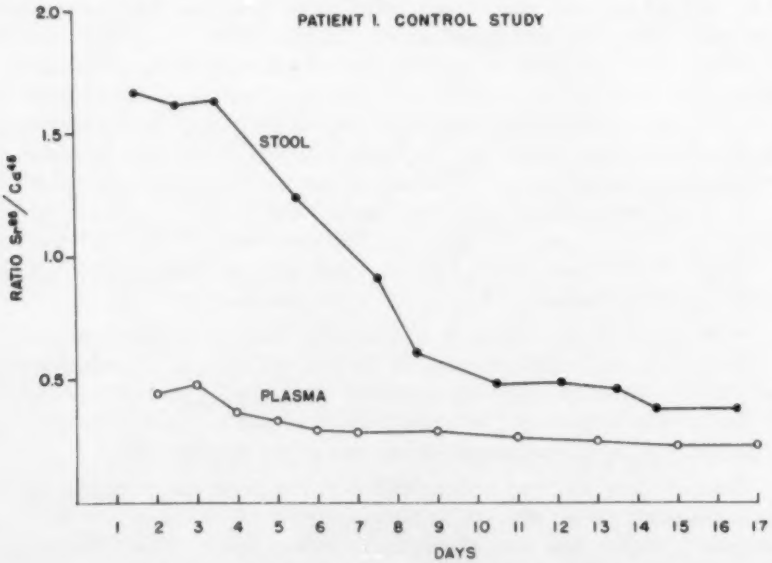

Fig. 4.—Ratio of Sr^{85}/Ca^{45} in stool and plasma.

TABLE II. URINARY AND FECAL EXCRETIONS OF CALCIUM, Ca^{45}, AND Sr^{85} IN THE THREE STUDIES OF SUBJECT 1

	STUDY 1 (CONTROL)						STUDY 2 (Na-EDTA)						STUDY 3 (Ca GLUCONATE)					
	URINE			STOOL			URINE			STOOL			URINE			STOOL		
DAYS	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)
1	3.25	1.63	92	6.85	4.09	251	1.03	10.24	510	39.17	21.97	323	10.50	8.10	437	0.03	0.03	34
2	1.44	0.77	64	7.61	4.71	46	0.75	0.23	27	–	–	–	1.43	0.92	79	10.72	4.84	129
3	1.31	0.76	89	49.81	30.58	315	1.95	0.80	88	35.41	19.59	168	0.89	0.55	56	61.41	30.17	243
4	0.92	0.55	77	–	–	–	1.28	0.54	81	–	–	–	0.97	0.52	66	6.31	4.54	190
5	0.70	0.55	76	–	–	–	0.97	0.41	70	7.00	4.81	263	0.94	0.69	97	–	–	–
6	0.84	0.67	115	10.67	8.73	418	0.89	0.41	78	0.73	0.75	125	0.73	0.62	95	0.33	0.48	54
7	0.69	0.51	110	0.47	0.53	122	0.68	0.36	72	0.34	0.43	102	0.71	0.58	100	0.84	1.55	236
8	0.44	0.37	83	0.28	0.45	96	0.75	0.41	96	0.48	0.66	143	0.48	0.44	76	0.43	0.87	161
9	0.45	0.42	95	–	–	–	0.56	0.32	80	0.20	0.29	81	0.50	0.47	97	0.47	1.19	284
10	0.28	0.27	68	0.18	0.37	189	0.38	0.22	63	0.25	0.38	104	0.32	0.33	73	–	–	–
11	0.27	0.28	89	–	–	–	0.46	0.28	87	0.31	0.50	177	0.34	0.30	73	0.23	0.54	143
12	0.27	0.27	82	0.57	1.15	396	0.41	0.28	84	0.23	0.35	118	0.33	0.28	73	–	–	–
13	0.24	0.25	88	0.11	0.24	86	0.31	0.24	83	0.18	0.34	103	0.31	0.28	78	0.30	0.74	211
Total	11.10	7.29		76.55	50.85		10.42	14.74		84.30	50.07		18.45	13.98		81.07	44.95	

TABLE III. URINARY AND FECAL EXCRETIONS OF CALCIUM, Ca^{45}, AND Sr^{85} IN THE THREE STUDIES OF SUBJECT 2

	STUDY 1 (CONTROL)						STUDY 2 (Na-EDTA)						STUDY 3 (Ca GLUCONATE)					
	URINE			STOOL			URINE			STOOL			URINE			STOOL		
DAYS	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)	Sr^{85} (% OF DOSE)	Ca^{45} (% OF DOSE)	Ca (MG.)
1	1.81	1.25	81	–	–	–	1.01	9.07	596	–	–	–	4.42	5.68	442	0.10	0.04	25
2	1.36	0.97	97	47.97	28.80	387	1.68	0.96	118	5.42	4.22	74	1.18	1.28	124	16.26	11.38	203
3	0.75	0.53	68	21.86	19.77	172	1.14	0.91	131	28.72	21.34	185	0.75	0.80	99	27.82	18.61	176
4	0.68	0.50	69	4.86	3.51	63	0.89	0.76	126	21.76	16.14	172	0.70	0.82	108	38.32	26.59	383
5	0.45	0.43	67	7.58	5.98	197	0.73	0.66	131	22.52	15.22	303	0.47	0.62	102	2.91	2.16	83
6	0.35	0.40	71	2.58	2.42	201	0.49	0.57	124	7.68	6.38	280	0.33	0.60	112	2.93	2.37	217
7	0.27	0.31	65	0.98	1.11	194	0.41	0.46	120	0.75	0.89	191	0.30	0.53	110	0.57	0.72	127
8	0.28	0.33	75	0.32	0.56	166	0.39	0.42	120	0.22	0.74	165	0.26	0.43	100	0.35	0.50	105
9	0.20	0.30	74	0.06	0.13	48	0.31	0.35	113	0.30	0.58	267	0.25	0.38	96	0.57	1.04	322
10	0.16	0.29	75	0.30	0.63	274	0.37	0.34	121	0.13	0.25	133	0.20	0.33	86	0.20	0.43	158
11	0.18	0.28	87	0.28	0.47	188	0.27	0.29	100	0.17	0.35	184	0.19	0.32	98	0.26	0.61	262
12	0.19	0.29	96	0.12	0.26	123	0.18	0.23	100	0.15	0.34	221	0.16	0.29	92	0.06	0.18	92
13	0.17	0.23	82	0.10	0.28	176	0.18	0.21	93	0.06	0.16	121	0.16	0.27	90	0.08	0.28	157
Total	6.85	6.11		87.01	63.92		8.05	15.23		87.88	66.61		9.37	12.35		90.43	64.91	

II. Na-EDTA Study.—The urinary calcium excretion rose markedly in both patients on the days of the Na-EDTA infusions, 510 and 596 mg. per day, respectively. The excess urinary calcium attributable to the administration of the chelating agent was calculated: 411 mg. for Subject 1, 509 mg. for Subject 2, corresponding to 63 and 78 per cent of the calcium binding power of the dose of Na-EDTA, respectively. The serum calcium of the first patient gradually decreased from 10.2 to 7.3 mg. per cent at the end of 6 hours of Na-EDTA infusion. In the second patient, serial determinations of serum calcium could not be performed. However, the serum calcium prior to the infusion of Na-EDTA was 10.1 mg. per cent, and 3 hours later it was 9.0 mg. per cent.

Metabolic balances: No significant changes of the calcium metabolism were noted (Table I).

Calcium^{45} study: The plasma Ca^{45} levels were slightly lower during the first days of Na-EDTA study than in the control period. The cumulative fecal Ca^{45} excretion was similar to that in the control study, 50.07 versus 50.85 per cent in Subject 1 and 66.61 versus 63.92 per cent in Subject 2. The rates of urinary Ca^{45} excretion of both patients increased greatly during the Na-EDTA infusion. They were approximately 20 times that of the control value at the end of 6 hours (Figs. 1 and 2). The 24-hour urinary Ca^{45} excretion of the 2 patients was approximately 6 and 7 times higher on the day of administration of Na-EDTA than in the control study. After the discontinuation of Na-EDTA the rate of Ca^{45} excretion decreased toward base-line levels.

Strontium^{85} study: No significant changes of plasma Sr^{85} levels occurred during or subsequent to the Na-EDTA infusions. The 13-day cumulative fecal Sr^{85} excretion of Subject 1 was slightly higher than in the control study while it remained unchanged in the second patient: 84.30 versus 76.55 per cent and 87.88 versus 87.01 per cent. On the day of the Na-EDTA infusions a significant depression of the rate of urinary Sr^{85} excretion occurred which was followed by a compensatory rise to levels higher than the control excretions (Figs. 1 and 2). Therefore, the cumulative urinary Sr^{85} excretion was similar in the Na-EDTA and control study.

Comparison of Ca^{45} and Sr^{85} metabolism: Profound alterations of the rate of urinary Sr^{85} and Ca^{45} excretions occurred during the administration of Na-EDTA. While the former was depressed to about one-sixth of the control level, the latter increased twenty fold. This relationship is illustrated by the ratio of Ca^{45}/Sr^{85} clearance (Fig. 3) which rises rapidly and reaches a peak at the completion of the Na-EDTA infusion. Shortly thereafter a precipitous drop of this ratio toward control levels was noted.

III. *Calcium Gluconate Study.*—The urinary calcium excretion increased on the day of the calcium infusion in both patients. This increment corresponded to an excretion of 56 per cent of the infused calcium. The serum calcium rose gradually and was 12.7 mg. per cent at the end of the 6-hour infusion in Subject 1 and was 14.3 mg. per cent after 4 hours of calcium infusion in Subject 2.

Metabolic balances: No significant changes of the calcium balances were noted (Table I).

Calcium^{45} study: The 13-day cumulative fecal Ca^{45} excretion of Subject 1 was slightly lower than in the control study, 44.95 versus 50.85 per cent, and remained essentially unchanged in Subject 2, 64.91 versus 63.92 per cent. The urinary Ca^{45} excretion was markedly elevated on the day of the calcium infusion in both patients; in Subject 2 control levels returned gradually while, in Subject 1, Ca^{45} excretion promptly reached base-line levels on the day after the calcium infusion (Tables II and III). The cumulative Ca^{45} excretion in 13 days was approximately twice as high as the control excretion in both patients.

Strontium^{85} study: The 13-day cumulative fecal Sr^{85} excretion was slightly higher in both patients as compared to the control study, namely, 81.07 versus 76.55 per cent and 90.43 versus 87.07 per cent. A marked rise of urinary Sr^{85} excretion was noted on the days of the calcium gluconate infusion. The cumulative urinary Sr^{85} excretions reflected this increase: 18.45 versus 11.10 per cent and 9.37 versus 6.85 per cent in the two patients, respectively.

Comparison of Ca^{45} and Sr^{85} metabolism: Although the urinary excretions of both isotopes Ca^{45} and Sr^{85} increased, Ca^{45} was preferentially cleared through the kidneys when stable calcium was infused (Fig. 3).

DISCUSSION

Regulation of calcium absorption from the intestinal canal in man to meet different physiologic and pathologic requirements and to balance the fluctuations of dietary calcium intake is one of the key problems of calcium metabolism. Several studies indicate that such an adaptive mechanism exists. The literature on this topic has been reviewed by Nicolaysen and associates.[6] The metabolic balance technique was employed in the study of this problem in subjects maintained on two or more calcium intake levels. The utilization of dietary calcium was estimated by relating improvements of calcium balances to the changes of the calcium intake. In a recent report from this laboratory data on the utilization of dietary calcium of two patients receiving 3 intake levels of calcium were presented. It was noted that endogenous fecal calcium measured by the radiocalcium technique did not increase significantly when the calcium intake was raised approximately tenfold.[10] It was concluded from these studies that the regulation of calcium absorption may play a major role in maintaining calcium homeostasis.[3]

It is generally assumed that the absorption of food calcium takes place in the uppermost segment of the gastrointestinal canal mainly in the duodenum and jejunum. Following the oral administration of Ca^{45}, radioactivity can be detected rather promptly in plasma, the maximum activity usually being reached 3 or 4 hours after the ingestion of the tracers. The experimental design of the study here reported was such that the plasma calcium levels were lowered or raised during 6 hours of the absorption of these tracers. It was hoped that major changes of intestinal absorption of calcium and of the tracers could be demonstrated during this stress. However, the fecal Ca^{45} and Sr^{85} excretions failed to show significant differences in the experimental studies, in spite of the fact that the calcium metabolism of the patients was definitely influenced by

the chelating agent and by the calcium infusions. These results indicate that acute changes of plasma calcium levels do not affect the intestinal absorption of calcium.

The effects of hypo- or hypercalcemia upon the metabolism of the *absorbed* fraction of the tracers were demonstrated in this study. As was previously shown from this laboratory the intravenous administration of Na-EDTA causes an increase of urinary calcium excretion as calcium is bound by the chelating agent in plasma and is rapidly eliminated as the calcium chelate by the kidneys.[15] The excess excretion of the chelated calcium corresponded to 63 per cent and 78 per cent of the binding power of the dose of Na-EDTA in the 2 patients. A marked rise of Ca^{45} excretion occurred concomitantly with the excretion of the calcium chelate. When the plasma calcium was raised by the infusion of calcium, the urinary Ca^{45} excretion was raised although to a lesser extent than after the administration of Na-EDTA. The excess urinary calcium excretion following the administration of ionic calcium was also less and was found to depend on the state of calcium metabolism of the subjects under study, whereas the excretion of chelated calcium seems to be independent therefrom.

The differences of intestinal absorption and urinary elimination of calcium and strontium previously described from this laboratory are confirmed in this study. The strontium absorption is about half as efficient as that of calcium, whereas the renal elimination of the absorbed strontium is 3 to 4 times higher than that of calcium.[11] The elevation of the serum calcium level significantly raises the urinary excretion of both ions, the excess excretion of calcium being somewhat higher than that of strontium.

The binding power of Na-EDTA is a hundred times higher for calcium than for strontium: $\log K_{Ca} = 10.6$; $\log K_{Sr} = 8.6$. It was reported from this laboratory that the urinary strontium[85] excretion decreases during Na-EDTA administration.[16] This observation was confirmed in both patients. However, in the present study employing the double tracers, the inverse relationship between the declining urinary Sr^{85} and the increasing Ca^{45} excretion was demonstrated. While the increment of Ca^{45} excretion is due to the excretion of chelated calcium the decreased urinary Sr^{85} excretion may be explained on the basis of increased tubular reabsorption of strontium which may accompany that of ionic calcium to maintain calcium homeostasis. The degree of this changing tubular resorption may be a sensitive indicator of parathyroid activity, secondary to changes of serum calcium levels. This change in parathyroid activity is indicated by the increase of urinary phosphorus excretion and decrease of serum inorganic phosphorus during the period of Na-EDTA infusion. Conversely, changes in opposite directions were repeatedly noted during calcium infusions.

When the Sr^{85}/Ca^{45} ratio of plasma was compared to that of stool, it was noted in both patients that these ratios form a parallel line after the unabsorbed tracers were eliminated from the intestinal canal (Fig. 4). However, the line representing the fecal Sr^{85}/Ca^{45} ratio was higher than that of the plasma. This was observed in all 6 studies which were performed in both patients. This indicates that more endogenous Sr^{85} is excreted with the digestive juices in the

intestinal canal or that more of the digestive juice calcium is re-absorbed. At present no definite statement can be made on the mechanism responsible for this observation.

SUMMARY

The effect of acute elevation or depression of serum calcium upon the intestinal absorption and upon the metabolism of the *absorbed* calcium and strontium was investigated in two patients.

The serum calcium was raised or lowered by a 6-hour infusion of calcium gluconate or sodium ethylenediaminetetraacetate. A tracer dose of Ca^{45} and Sr^{85} was administered orally at that time.

Acute changes of the serum calcium levels had no significant influence on the absorption of either calcium or strontium; the absorption of Ca^{45} exceeded that of Sr^{85} under all conditions studied.

The urinary excretion of Sr^{85} exceeded considerably that of Ca^{45} in the normocalcemic state. During hypercalcemia the excretion of both tracers increased; ethylenediaminetetraacetic acid caused a marked increment of Ca^{45} and a marked decrease of Sr^{85} excretion.

The endogenous fecal excretion of Sr^{85} was slightly higher than that of Ca^{45} in all studies.

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INFLUENCE OF CHELATES ON THE METABOLISM OF RADIOYTTRIUM (Y^{90}). III

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IN PREVIOUS studies with rats and rabbits it was shown that the site of deposition of intravenously administered yttrium could be influenced by both the chemical nature of the chelating agent and its concentration.[1] Further studies with rabbits and dogs have shown that the biologic fate of Y^{90} is markedly influenced by time and the dose of carrier (as mg. Y^{89} per kilogram body weight).[2] Under the conditions of the experiment the greatest concentration of this metal (as micrograms yttrium per gram of fresh tissue) was found in trabecular bone and stomach mucosa. On the basis of these findings it was suggested[1, 2] that the selectivity for these tissues, might be useful as a means of delivering the high-energy betas of Y^{90} to neoplastic lesions involving these tissues. One purpose of the present investigation was to further explore these possibilities.

By selecting those conditions which previously were found to favor the deposition of yttrium in bone (sparing the liver, spleen, and stomach mucosa where possible) the beta emission of Y^{90} has been utilized as a means of inducing controlled hematologic changes in rabbits and dogs.[3] Details of these results are given in this report.

In the present paper are also given results of the autoradiographic study of the localization of Y^{90} in normal and healing bone as well as the distribution of yttrium in 3 patients, utilizing Y^{90} as a tracer, chelated with an excess of *N'*-hydroxy ethylenediamine triacetic acid (ED-ol).

MATERIALS AND METHODS

Preparation of Yttrium Solution.—Because of the need for high specific activity Y^{90} in these studies, sterile solutions containing approximately 10 mc. Y^{90} per milliliter with 0.2 mg. Y^{89} per milliliter as carrier were obtained from the Abbott Laboratories, Oak Ridge, Tenn. This material is prepared by selectively leaching the Y^{90} from a resin column containing an equilibrium mixture, $Sr^{90} \rightarrow Y^{90}$.

A solution of $Y(NO_3)_3 \cdot 5\ H_2O$ was prepared in sterile, pyrogen-free water; a drop of alcoholic phenolphthalein was added and then sufficient *N'*-hydroxy ethylenediamine triacetic acid was added to give a permanent pink color. The solution was adjusted so that final concentration was 1.0 mg. Y per milliliter (pH 8 to 9) and autoclaved to ensure sterility.

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In preparing an injection, that volume of Y^{90} solution was determined which would yield the required millicuries Y^{90} per kilogram of body weight. The "carrier" dose (Y^{89}) was found by determining the amount of Y^{89} accompanying the Y^{90}, then calculating the number of milliliter of the chelated carrier necessary to give the required milligrams of Y^{89} per kilogram.

The Y^{90} solution was introduced into a sterile syringe of suitable size, and sufficient carrier solution then drawn into the syringe. With some air space within the syringe, the two solutions were mixed and the air expelled. A pink solution (pH 8 +) resulted since the quantity of acid and metal in the Y^{90} solution was insufficient to markedly affect the final pH.

Preparation of Autoradiographs.—Sterile chelated Y^{90} solutions containing 2.0 to 5.0 mc. Y^{90} with carrier in the range of 1.0 to 2.0 mg. Y^{89} per kilogram were injected into the marginal ear vein of albino rabbits. The animals were sacrificed at the end of a 24-hour period and various bones removed. The excess soft tissue was cleaned off, and the bones imbedded in a plaster of Paris mixture (quick setting dental stone). In one to two hours these mountings were cut in half, using a metal cutting band saw, and the surface was ground smooth on fine sand paper, laid on a flat surface. The mounts were washed with cold water, using a fine brush to clear debris from the interstices of the bone. The smooth surface of the mounted bone was then covered with thin aluminum foil acting as a moisture seal, and then clamped in close contact with the emulsion side of fine-grain 35 mm. panchromatic film. Suitable exposure times were determined by trial, and development of the exposed film with a suitable fine-grain developer. These autoradiographs gave an excellent indication of those areas in the bone in which yttrium is concentrated.

Deposition of Y^{90} in Healing Fractures.—In order to determine the degree of deposition of yttrium in areas of bone having high osteoblastic activity, one rib of each of 4 young albino rabbits (2.5 to 3.0 kilograms) was fractured while the animals were under general anesthesia. In 14 days excellent callus formation was evident and each animal received, intravenously, into a marginal ear vein, 2.0 mc. Y^{90} per kilogram and a carrier dose of 1.0 mg. Y^{90} per kilogram of body weight. The animals were sacrificed 48 hours after the Y^{90} injection and the ribs removed. The concentration of Y^{90} in the healing fracture was compared with the Y^{90} of the opposite normal rib by the counting procedure outlined below. Autoradiographs were prepared of the fractures as described above.

Induced Hematologic Changes.—In order to determine the lethal and sublethal dose range of Y^{90} due to radiation effects, young adult albino rabbits (2.5 to 3.0 Kg.) were injected, in the marginal ear vein, with solutions of Y^{90} chelated with *N'*-hydroxy ethylenediamine triacetic acid (ED-ol) at 1.5 to 4.5 mc. Y^{90} per kilogram and carrier of 0.05 to 1.5 mg. Y^{89} per kilogram. This carrier dose range has no toxic effects, and the metal alone has produced no observable hematologic changes. A total of 8 rabbits were used in this series of experiments.

Mongrel dogs (8 to 12 kilograms) were given Y^{90} at 0.50 to 1.5 mc. Y^{90} per kilogram with carrier of 0.05 to 1.5 mg. Y^{89} per kilogram. These 18 dogs were administered the material by injection into one of the superficial veins of the hind leg.

Peripheral blood samples were drawn from the opposite ear and leg, respectively, of each animal before the injection and at 48-hour intervals thereafter for 8 days, after which time weekly blood samples were taken. Total erythrocyte and leukocyte counts were made, and the cellular differential, hematocrit, and hemoglobin content determined.

In several of the dogs when the leukocyte count was reduced below 2,000 cells per cubic millimeter, a broad spectrum antibiotic was injected intramuscularly to reduce the mortality from generalized infection. In some instances this treatment seemed to produce favorable results, and the animal survived an otherwise lethal radiation dose.

Distribution of Y in Man.—In order to determine the distribution of Y in man and to compare it with that found in animals, 6 terminal patients were administered, intravenously, 2.0 mc. Y^{90}, chelated with ED-ol in a carrier dose of 0.05 to 0.20 mg. Y^{89} per

kilogram. Autopsies were obtained in 3 of these patients, at which time representative samples of various tissues were removed, and the relative Y^{90} content determined by the counting procedures outlined below.

Analytic and Counting Procedures.—To determine the relative Y^{90} content of tissues, two thinly sliced 2 to 5 Gm. representative samples of each fresh tissue were taken from each organ, weighed into tared planchets, dried for 12 hours at 120° C., again weighed, and the beta emission determined by counting with an end-window Geiger tube (3 mg./cm.^{2} mica; background 25 to 30 c.p.m.; tube to sample distance, 10 mm.). The observed counts were corrected for decay ($t_{1/2} = 61$ hours) to some common time, usually the time of death. The counts so obtained yielded the net counts per minute per gram of fresh tissue (error ± 5 per cent). These values are an index of the Y^{90} content and are semiquantitative in nature, since no correction was made for selfabsorption by the samples.

To obtain a quantitative estimation of the yttrium content of tissues, aliquots of the solutions which were injected were mixed with lean beef; 2 to 5 Gm. of these samples were weighed, dried, again weighed, and the net counts per microgram of added yttrium determined. Under these conditions the net counts obtained from Y^{90} were a linear function of the added yttrium (Y^{89} + Y^{90}) in the counting range (1,000 to 4,000 c.p.m.).

Beta decay and absorption studies have been made, utilizing many Y^{90} samples, both those prepared from Sr^{90} by the Abbott Laboratories and those prepared by neutron activation of Y_2O_3. These samples were studied by Geiger counting of beta-gamma emission for up to 120 days. It was found that 6 different lots of the Abbott material contained 1 part Sr^{90} in 10^7 to 10^8 parts Y^{90}. This material is obtainable with relative low-carrier content (circa 0.02 mg. Y^{89} per millicurie Y^{90}) and since it is necessary to rigidly control the carrier dose, this commercially available material was utilized throughout the pharmacologic investigations reported herein.

Samples of commercially "pure" Y_2O_3, as well as those further purified in this laboratory have been neutron activated and all have shown appreciable quantities of other rare earths. The principal contaminants appeared to be Tb^{160}, Er^{169}, and Yb^{175} which were present in combined amounts of the order of 1 part in 10^3 of Y^{90}. The highest purity Y_2O_3 obtained was that kindly furnished by Professor F. H. Spedding of the Institute for Atomic Research, Ames, Iowa.

RESULTS

Lethal Dose and Hematologic Studies (Rabbits and Dogs).—

Rabbits: Examination of the results of the studies of 8 rabbits intravenously injected with Y^{90} chelated with ED-ol shows (a) that the lethal range is within 3.5 to 4.5 mc. of Y^{90} per kilogram and (b) that the hematologic changes in the sublethal dose range, 1.5 to 2.5 mc. Y^{90} per kilogram, are comparable with those found in whole body x-radiation, except that the effects on the erythrocytes are somewhat more transitory.

In the 4 rabbits receiving 1.5 to 2.5 mc. Y^{90} per kilogram, the total leukocyte count dropped from a mean value of 7,900 cells per c.mm. to 4,900 cells per c.mm. in 2 to 5 days and remained at this figure for the next 3 weeks, after which time normal counts were obtained. Normal erythrocyte counts (5.1×10^6 cells per c.mm.) were maintained for 7 days, following the administration of the Y^{90} at which time they fell to 4.2×10^6 cells per c.mm. Normal values were again observed within 14 days. These latter animals were normal in appearance throughout this series of blood studies. Further details are omitted since the results of the hematologic studies with dogs confirm and extend these findings.

Dogs: Intravenous administration of Y^{90} to mongrel dogs has shown that none of the 8 given 1.5 to 2.5 mc. Y^{90} per kilogram survived. All died within 21 days with evidence of generalized internal hemorrhage. However, 3 of 6 survived in those dogs receiving 1.0 mc. Y^{90} per kilogram, with all being given a broad spectrum antibiotic when the total leukocyte count fell below 2,500 cells per c.mm. At 0.5 mc. Y^{90} per kilogram no significant effects other than hematologic changes were observed in the 4 dogs tested.

In the dogs receiving 1.0 mc. per kilogram the initial drop in the total leukocyte count was accompanied by essentially complete disappearance of platelets, with an increase in the hemoglobin from a mean value of 14.5 Gm./100 ml. to 18.0. Hemoconcentration was indicated by a simultaneous increase in the hematocrit from 0.45 to 0.53 in the 5 days immediately following the administration of Y^{90}. By the end of 45 days thereafter, the hemoglobin and hematocrit had returned to normal levels.

The mean neutrophil/lymphocyte ratio for the 3 animals which survived 1.0 mc. Y^{90} per kilogram dropped from an initial 2.2 to 0.34 in 14 days. This reversal of the normal persisted for an additional 16 days after which time the ratio began to rise, reaching the initial value 40 days after the injection of the Y^{90}.

In the 4 dogs receiving 0.5 mc. Y^{90} per kilogram with carrier of 0.05 mg. Y^{89} per kilogram, the initial drop in the total leukocyte count was accompanied by essentially complete, but quite transitory, disappearance of platelets. In contrast with the higher dose there was no hemoconcentration as the hematocrit fell from an initial 0.47 to 0.34 on the twenty-fifth day postinjection. Thereafter, the hematocrit rose, reaching the initial value on the sixty-seventh day following administration of the Y^{90}. The change in hematocrit was paralled by a drop in the hemoglobin content of the whole blood from an initial value of 13.0 Gm. per cent, to 10.0 on the twenty-sixth day postinjection. A normal value (13.1 Gm. per cent) was observed on the sixty-seventh day after administration of the Y^{90}.

The mean neutrophil/lymphocyte ratio for the 4 dogs receiving 0.5 mc. Y^{90} per kilogram gradually dropped from an initial 2.7 to 0.52 on the twenty-sixth day after administration of the $Y.^{90}$ This ratio then increased slowly so that a normal ratio (2.9) was not obtained until 96 days following Y^{90} injection. These findings are similar to those of the 3 dogs surviving double this dose; however, the changes were slower in appearing and persisted for a somewhat longer time.

Fig. 1 graphically illustrates the effects on total erythrocytes and leukocytes of the intravenous administration of chelated Y^{90} to mongrel dogs. The results shown are the mean of the observed counts.

At a dose of 1.0 mc. Y^{90} per kilogram, the number of leukocytes were reduced 82 per cent in 14 days in *those animals which survived.* Concurrently, the erythrocytes underwent a slow decline, never more than 15 per cent below the initial level. The leukocytes returned to the initial level at 50 to 60 days

following the injection of Y^{90}. The number of erythrocytes remained depressed to the end of the 84-day observation period. In those dogs which died as a result of this dosage, the total leukocytes were reduced to less than 1,000 cells per c.mm. No significant effect was observed in the number of erythrocytes in these animals since death usually ensued within 14 days after injection.

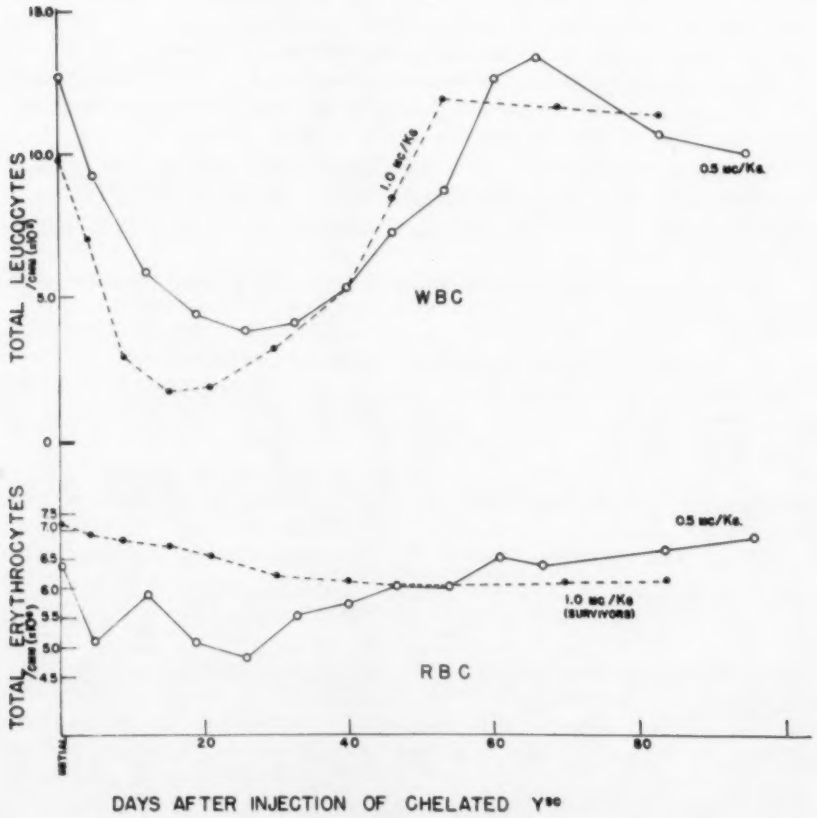

Fig. 1.—Influence of intravenous chelated Y^{90} on peripheral blood of dogs.

At a dose of 0.5 mc. Y^{90} per kilogram, the number of leukocytes were progressively reduced 70 per cent in 26 days following the injection. These values are the mean of *all dogs so treated* and, therefore, represent no natural selection of those most resistant, as in the case of the 3 dogs shown for the 1.0 mc. per kilogram dosage. At 0.5 mc. per kilogram the number of erythrocytes were reduced 25 per cent below the initial value, paralleling the drop in the leukocytes during the first 26 days postinjection. All formed elements of the

blood returned to the initial levels by the sixtieth day postinjection and remained at normal levels to the end of the 96-day observation period.

Autoradiographic Studies.—Examination of contact autoradiographs prepared from the bones of young adult rabbits 48 hours following intravenous administration of chelated Y^{90} show that this metal is concentrated at the endosteal and periosteal surfaces, surfaces of the haversian canals, the trabeculae, and at the epiphyseal junction. These findings indicate a predilection for those areas having marked osteoblastic activity and are in excellent agreement with the findings of Jowsey and associates[4] when using the longer life Y^{91}.

The autoradiographs are not reproduced here since they are indistinguishable from many previously published, particularly those by Jowsey and associates[4] and those for Ga^{72} by Dudley and co-workers.[5,6]

Yttrium in Healing Fractures.—The concentration of Y^{89} and Y^{90} in areas of osteoblastic activity is further indicated by the results found on the determination of Y^{90} content of the healing fractured ribs of young adult rabbits (Table I).

Autoradiographs confirm these findings, indicating a zone of concentration within the callus.

TABLE I. YTTRIUM DEPOSITION IN HEALING FRACTURES*

TISSUE SAMPLE	RELATIVE CONCENTRATION OF YTTRIUM (NET COUNTS/MIN./GM. FRESH TISSUE [$\times 10^3$])
Normal rib	15.9
14-day callus	23.7

*Dose was 2.0 mc. Y^{90} per kilogram with a carrier content of 1.0 mg. Y^{89} per kilogram given intravenously as the chelate to each of 4 rabbits. Animals were sacrificed 48 hours after injection.

TABLE II. AUTOPSY FINDINGS: RELATIVE CONCENTRATION OF YTTRIUM IN FRESH TISSUES (SKELETAL MUSCLE = 1)

	TISSUES									
PATIENT*	LIVER	KIDNEY	SPLEEN	TRABECULAR BONE	MARROW	WHOLE BLOOD	HEART	STOMACH MUCOSA	LUNG	COLON
W. V. (M 76) cirrhosis with ascites (4 hr.; 0.05 mg.)	6.0	2.5	2.9	21.9	1.4	1.4	1.4	7.0	6.0	1.4
M. B. (F 60) hyperneph-roma (5 days; 0.20 mg.)	32.2	13.3	25.6	4.2	Trace	Trace	11.9	5.2	2.6	1.5
J. M. (F 35) adeno ca. ovary (11 days; 0.20 mg.)	17.4	10.2	16.7	8.9	Trace	Trace	5.6	7.5	4.1	2.6

*Initials, sex, age, diagnosis, time of death after I.V. administration of Y^{90}, carrier dose (mg. Y^{89} per kilogram of body weight).

Distribution of Yttrium in Man.—In Table II are shown the details of the findings in the study of distribution of Y in 3 patients who were administered intravenously 2.0 mc. Y^{90} chelated with ED-ol. Because of the varying time of

death, general condition, and the difference in carrier dose, the results are preliminary in nature. However, they are in general agreement with those previously found with rabbits and dogs.

At the low carrier dose (0.05 mg. Y per kilogram) yttrium is quickly deposited in bone and the stomach mucosa. However, at a carrier level of 0.20 mg. Y per kilogram, the deposition is more marked in the liver, kidney, and spleen.

DISCUSSION

The experiments outlined in this paper were undertaken as a final preliminary step in the evaluation of the potential usefulness of systemically administered radioyttrium (Y^{90}) in the therapy of neoplastic conditions involving the bone and/or marrow in man.

The findings confirm and extend data previously obtained by the authors[2] which show that Y^{90}, chelated with an excess of N′-hydroxy ethylenediamine triacetic acid (ED-ol) and at a low carrier dose ($\sim$ 0.05 mg. Y per kilogram body weight) will localize quite selectively in the skeleton. The energetic beta emission (maximum 2.2 mev.) irradiated the adjacent marrow, causing selective destruction, or at least a marked reduction in its potential to produce immature or bizarre forms of the cellular elements of the blood. The relatively short half-life of the Y^{90} permits good control of radiation dosage. A further safeguard is the ease with which yttrium may be cleared from the body should too high a dose level be established. Thus, intravenous administration of a chelate such as Ca-EDTA, a standard pharmaceutical preparation, promotes rapid clearance of yttrium via the kidney.

The data reported in this study demonstrate that with careful titration of the dose of Y^{90} administered it is possible to effect preferentially the various components within the marrow and hence the peripheral blood. It is also possible that neoplasms involving the bone (primary or secondary) may be effected in a salutary manner with the proper manipulation of the dose of Y^{90} given.

Following this line of reasoning, clinical trials have been undertaken in a variety of neoplastic conditions involving the marrow and/or bone, such as leukemia (chronic and acute), polycythemia vera, multiple myeloma, and various bone tumors of 1 and 2 degrees. Details of these studies will be presented in the next paper of this series.

The use of ED-ol in both the animal and clinical studies stems from the results of our earlier screening of various chelates.[1] This does not preclude the possibility that other more useful complexing materials may be found. The rapid development of chelation chemistry may well yield compounds which will permit very precise control over the "nonphysiologic" metals when used in biologic material for a variety of purposes.

SUMMARY AND CONCLUSIONS

Studies with rabbits and dogs following the systemic administration of radioyttrium (Y^{90}) chelated with ED-ol, at low carrier concentration, have

shown that this metal may be caused to localize in bone, particularly in those areas having marked osteoblastic activity. The energetic beta emission irradiates the adjacent marrow. Titration of the dose of Y^{90} given resulted in a preferential effect on the components of the marrow and, hence, the peripheral blood. The lethal dose for rabbits was found to be 3.5 to 4.5 mc. Y^{90} per kilogram and for dogs, 1.5 to 2.0 mc. per kilogram. Sublethal doses produced typical radiation effects on the peripheral leukocytes (70 to 80 per cent reduction). The number of erythrocytes were not markedly reduced under these conditions. Control levels were regained by the leukocytes within 60 days after the Y^{90} injection.

Chelated Y^{90} (2.0 mc.) was administered intravenously to 6 terminal patients, 3 of whom came to autopsy. In the 2 patients given a carrier dose of 0.20 mg. Y^{89} per kilogram the major localization was in the liver and spleen, whereas the patient given a carrier dose of 0.05 mg. Y^{89} per kilogram showed significant localization in bone.

Based on the results obtained in this study it is suggested that Y^{90} ED-ol, at low carrier concentration, may be useful in the management of malignant conditions involving the marrow and/or bone. Clinical trials of this modality are now actively underway in a wide variety of such conditions.

We wish to acknowledge with thanks the assistance rendered by Billy J. Boaz (HM1), USN, throughout this entire investigation; we also thank Captain S. S. Sarkisian (MC), USN, and his staff of the Pathology Laboratory for the many blood studies, as well as the autopsy material.

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KINETIC STUDIES OF SKELETAL METABOLISM BY EXTERNAL COUNTING OF INJECTED RADIOISOTOPES: THE RADIOISOTOPE OSTEOGRAM

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A NUMBER of tests involving radioisotopes are well established for diagnosis of malfunctions of the human thyroid gland, kidneys, and liver. One general line of development has been toward measuring changes in radioactivity within the body by using detectors held near the skin and pointed at various parts of interest. Techniques of this sort yield data concerning the dynamic behavior of the radioactive material. Such information is often unobtainable from assays of daily excreta. The purpose of the work now to be reported was to explore certain of the less well-known aspects of bone physiology with external counting methods. It is believed that these animal experiments in this field will form the basis for clinical procedures having sound and immediate medical utility.

Calcium is, of course, only one of many substances absolutely vital to bone tissue. However, a slight disturbance of the nice balance of calcium flow into and out of bone can have profound and readily observable effects. It is a simple matter to administer radioactive calcium[45] and thus to add distinguishable "labels" to this flux. Therefore, an extensive literature has evolved, dealing with those facets of skeletal metabolism which involve calcium.[1, 2] A great deal is already known of the modes of transport and ultimate fixation of calcium. The present studies, however, are primarily concerned with events during the *first hour* after injection of radioactive materials which find their way into normal bone tissue. The experiments also disclose how abnormal conditions can alter these events. The low penetrating power of the beta rays of Ca^{45} make is unsuitable for measurement by detectors placed outside the body. However, cyclotron-produced Sr^{85}, which emits only gamma rays, is commercially available, and serves admirably as a substitute label for calcium movements, providing certain metabolic differences are reckoned with.[3, 4]

MATERIALS AND METHODS

Female Dutch rabbits of various ages were used throughout these experiments. Most of the data appeared in the form of strip chart recorder tracings which indicated the variations of radioactivity (counts per minute) in a specific body area as a function of time after intravenous injection of the radioactive material.

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Carrier-free Sr^{85} was purchased from the Nuclear Science and Engineering Corporation; $Na\ I^{131}$, Na^{24}, and Ba^{140}-La^{140} were obtained from the Oak Ridge National Laboratory; and Na^{22}, Cr^{51}, and radioiodinated human serum albumin (RISA) from Abbott Laboratories. Doses ranged from 5 to 20 μc except for long-term retention studies, in which 50 to 75 μc were administered.

The basic equipment consisted of a lead shielded, collimated, gamma scintillation detector probe with a 1 by 1 in. sodium iodide crystal, photomultiplier tube, and preamplifier whose output was fed to a counting ratemeter. After immobilizing the rabbit by taping the extremities to a board, the detector was clamped in an adjustable, counterbalanced holder and placed in contact with the leg bone or other part of the body being studied. Intraperitoneal injections of urethane were used to sedate the rabbits and minimize struggling during long runs.

After the background counting rate registered by the ratemeter (Nuclear-Chicago 1620 or Tracerlab Precision) had been recorded on the strip chart recorder (Esterline-Angus) for an adequate period, the radioisotope was injected into an ear vein, and the fluctuations of radioactivity (counts per minute) in the field "seen" by the detector were recorded for the desired period.

An important refinement of the method was the use of a gamma spectrometer (single channel pulse height analyzer, Nuclear-Chicago 1810) between the detector and the ratemeter. The heights of the voltage pulses emitted by the photomultiplier are proportional to the energies of the nuclear radiations striking the crystal. With a pulse height analyzer one can sort these pulses and choose for recording any desired range. Thus, one may discriminate against low energy pulses due to instrumental noise and Compton scattering and record only those gamma photons having energies lying within the chosen "window." Many different arrangements of these instrumental units are possible. In several experiments, the electrical output of a single detector aligned over a leg bone, for example, was split and sent to *two* single channel analyzer-ratemeter-recorder combinations. The behavior of each radioisotopic species in a mixture of two gamma emitting radioisotopes could then be recorded separately by proper adjustment of each pulse height analyzer. In other experiments, one detector-analyzer-ratemeter-recorder combination was set to record events in one leg and a second, similar but separate combination, was adjusted to record events at some other location.

For those instances when circulating blood was the object of study, both ends of a polyethylene plastic cannula, 24 inches long and 0.060 in. outside diameter, were inserted and tied into one of the carotid arteries. This operation was performed on heparinized rabbits, under ether anesthesia. Prior to insertion, the cannula was filled completely with heparin and saline solution to exclude air bubbles and prevent clotting. An external loop of circulating blood was thus obtained. A coil of this loop was taped over the shielded crystal of one detector probe, placed as close as possible to the neck. Only about 1 out of every 3 attempts at this operation was successful, but the effort was necessary to obtain valid, continuous tracings of blood clearances of radioisotopes, without repeated withdrawing of samples by hypodermic syringe.

RESULTS AND DISCUSSION

Initial Phases of Radiostrontium Uptake.—Fig. 1 illustrates a typical tracing following an intravenous Sr^{85} injection. One detector probe was aligned over the tibia midshaft and a second detector-recorder combination was arranged to record the blood activity in a carotid cannula loop in the same animal. These are facsimilies traced from the actual penned records, with the ruled coordinate lines of the chart paper omitted. Horizontal base line marks are 5-minute intervals. The time of injection is marked A. As time, postinjection, increases, the trace progresses toward the left. Marks on the curved vertical axis are references for obtaining the counts per minute at any time during the experiment. The greater the amount of radioisotope in the field of view of

the detector, the higher the trace. The flat trace prior to injection is the background counting rate. The shape or trend of the record is one of its most important features. The *change* of radioactivity with the passage of time is often of greater significance and utility than the static measurement of the absolute amount of radioactivity present at any one time. Furthermore, it would be extremely difficult to ascertain the absolute activities (disintegrations per second) in the tissue fields "seen" by the detectors, because of unknown factors of geometry and scattering. In this rabbit, there was a rapid build-up of activity in the tibia midshaft area which reached a maximum at about 10 to 13 minutes, after which the level remained practically constant for at least another 40 minutes. Activity in the blood reached a maximum within one minute after injection, then fell precipitously. Plotting the values taken from several such records indicated that neither the bone nor the blood traces are simple exponential functions, but probably reflect the concurrent operation of at least 3 processes which will come under discussion later.

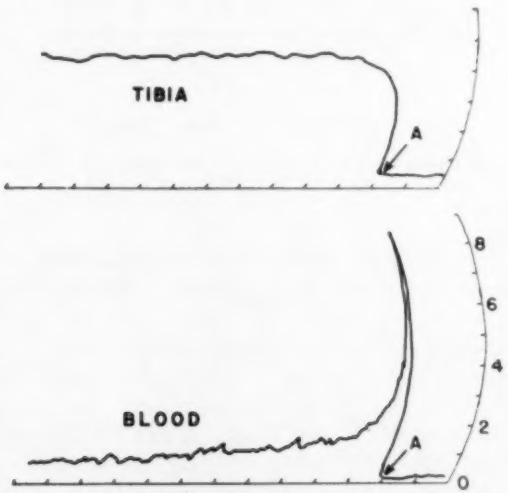

Fig. 1.—Bone pickup and clearance of Sr^{85}.

For adult normal rabbits, the time required for the tibia midshaft to attain maximum activity following an intravenous injection of Sr^{85} chloride, was usually between 15 and 35 minutes. Fig. 2 gives the distribution of (T_N) values for a series of 33 rabbits. It was found that the times to reach maximum activity in normal bones (T_N) were in general, greater for bone areas containing spongiosa than for fields primarily made up of dense cortical bone. Thus a knee or elbow trace will often continue to rise for perhaps 15 to 45 minutes after the trace for the tibia midshaft of the opposite leg has reached its peak and leveled off. The ratio of T_N for knee to T_N for tibia shaft was greater than one and was greatest for very young growing rabbits. The usefulness of comparing T values for abnormal and normal bone areas is developed in later paragraphs concerning healing fractures.

It was instructive to compare the disappearance of Sr^{85} from the blood with its appearance in bone. In the particular experiment shown in Fig. 3 one counting unit was set to record circulating blood activity in a cannula loop and a

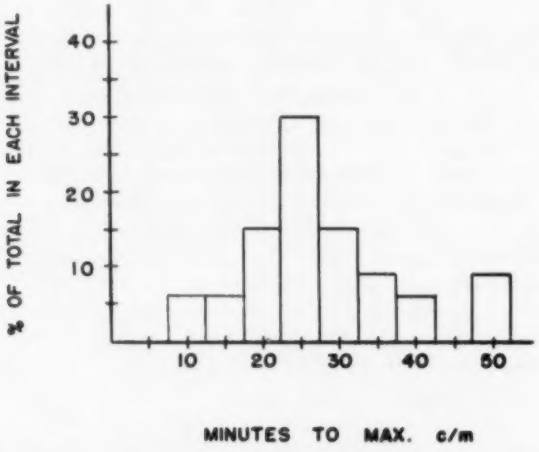

Fig. 2.—Distribution of times required for maximum Sr^{85} uptake in midshafts of tibiae of 33 normal rabbits.

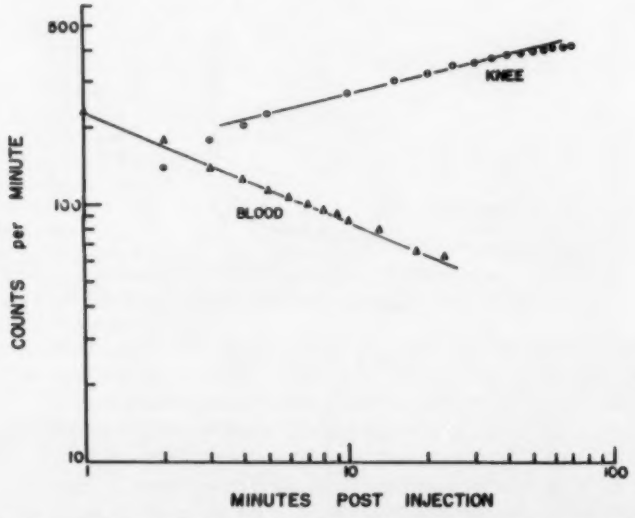

Fig. 3.—Disappearance of Sr^{85} from blood and accumulation in knee area, recorded simultaneously following intravenous injection.

second unit was aligned over the left knee. The blood activity reached its peak within one minute after the injection, but about 70 minutes were required for the knee field. When the logarithms of observed activity were plotted against

the logarithms of elapsed time, a straight line fitted most of the blood disappearance data fairly well. Thus, the activity could be described in terms of a negative power function of time. (Activity) = (a) $(\text{time})^{-b}$, where a and b are constants. The knee data for activity appeared to fit a positive power function of time, but only for the 5- to 30-minute interval. The slopes of the two lines are, of course, opposite in sign. The rate of appearance in this knee did not exactly match the rate of disappearance from the blood.

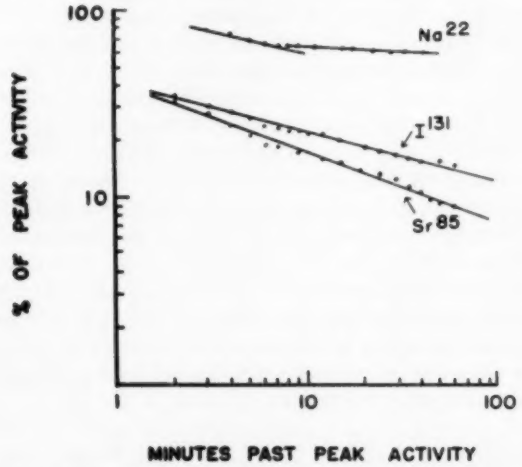

Fig. 4.—Clearance of Sr^{85}, I^{131}, and Na^{22} from blood circulating in a plastic tube inserted in a carotid artery.

The rapid clearance of Sr^{85} from the blood is very likely a consequence of its fixation by bone tissue. Fig. 4 shows how the rate of Sr^{85} disappearance from blood circulating in a cannula loop exceeds that of iodide ion (Na I^{131}) and sodium ion ($Na^{22}Cl$). The data from these 3 experiments is also plotted on log-log coordinates because in this manner a straight line describes the observed trends fairly well. The slope for Sr^{85} clearance is steeper than for either iodide or sodium ion. Such log-log or power function plots are very useful for empirical descriptions and comparisons of trends.[5, 6] However, they do not permit mathematical analyses of the data in terms of a series of exponential functions to each of which some distinct physiologic process can be assigned. A number of such detailed analyses of blood disappearance curves for calcium, chloride and sodium ions have been published.[7-13] For the purposes of the present paper, however, it is sufficient to point out the usefulness of gamma counting of an external loop of circulating blood in such studies. This technique has been applied by MacIntyre and Leonards to several gamma-emitters.[14]

Let us now attempt to interpret the bone records in terms of the physiologic events which transpire during the first hour following Sr^{85} injection. Before proceeding, it must be recognized that the field seen by the collimated detector

probe includes not only bone tissue but also considerable blood, lymph, skin, muscle, and adipose tissue. The activity-time record is the summation of fluctuations of activity in each of these compartments. The movements of Sr^{85} into and out of each of these "spaces" can at present be described only in rudimentary fashion. After injection into a vein, the circulation carries the radioactive material into the bone area via the capillary network. Thereupon, diffusion or transport across the capillary membrane permits the radioisotope (probably ionic, in the case of $Sr^{85}Cl_2$, $Na^{22}Cl$, and Na I^{131}) to permeate the tissues in the area. Transport into the bone itself is probably by way of the haversian canals, Volkmann's canals, and canaliculi. Fixation in the bone can then occur by exchange with an equal number of nonradioactive ions already at the spot, by accretion of new bone salts, and by binding with organic components of the bone matrix in which the bone salt crystallites are enmeshed.[2] The actual shape of the activity-time record is thus determined by: the blood mixing time; the rates of diffusion out of and back into the capillaries; the size of the capillary network (i.e., the vascularity); the architecture of the bone area (e.g., cancellous, with many trabeculae or compact, with well-defined haversian systems); and the metabolic status of the bone area (e.g., in active ossification and growth or in a pathologic resorptive condition). In addition to these physiologic factors, the instrumental lag in response, due primarily to the counting ratemeter time constant which is selected, also affects the shape of the record somewhat. This is particularly true when the activity level is changing rapidly, as in the first few minutes after injection.

Fig. 5.—Behavior of Cr^{51}-tagged red cells and Sr^{85} in normal rabbit knee.

It is possible to estimate the contributions of vascular transport to the observed activity-time records. For example, the blood clearance studies using the cannula loop indicated that the blood circulating through a carotid artery reached its maximum activity within a minute after injection. Four experiments in which red cells, tagged with Cr^{51} by the method of Gray and Sterling,[15] were injected showed that the red cells were uniformly mixed in the skin-muscle blood-bone area seen by the detector within 3 to 8 minutes. Fig. 5 shows the record obtained from a single detector aligned over a knee. Cr^{51}-tagged red cells were injected into one ear vein, with the pulse height analyzer adjusted to record Cr^{51} gammas (0.32 mev.). After about 55 minutes, without disturbing the position of animal or detector probe, the analyzer window was reset to record only Sr^{85} gammas (0.51 mev.) and Sr^{85} was then injected into the other ear. Red cell activity reached a plateau in about 7 minutes whereas Sr^{85} activity was still increasing in the area 60 minutes after its injection. It would seem therefore, that the speed of mixing and transport within the vascular network is rapid enough to ensure that the maximum concentration of Sr^{85} inside these

capillaries in a bone area is reached well within 8 minutes. The continued increase in Sr^{85} activity observed after this period is evidence for the operation of one or more of the other processes mentioned above.

Proceeding now to an assessment of the contribution of transcapillary movement to the observed activity-time records, the results of a number of experiments with radiosodium will be cited. Na^{22} was used extensively in these animal experiments because of its low cost and long half-life (2.6 years). A positron is emitted during its decay, the annihilation of which produces two gamma photons whose energies happen to be the same as that of Sr^{85} gammas (0.51 mev.). Therefore, in double tracer experiments it was customary to inject Na^{22} first, followed by Sr^{85} only after a plateau of Na^{22} activity had been established on the record. However, in 5 experiments, the behaviors of both sodium and strontium in the same bone area were recorded simultaneously. A solution containing both Sr^{85} and Na^{24} (15 hr. half-life) was injected and the output of a single detector, aligned over the desired area, was sent to two analyzer-recorder circuits. One analyzer was adjusted to record only the Sr^{85} photons entering the detector and the other to record only those photons having the energy range characteristic of Na^{24} (1.4 mev.). In general, the time required for maximum radiosodium activity to appear in bone areas was longer than that for Cr^{51}-tagged red cells, but shorter than that for Sr^{85}. Fig. 6 presents the time intervals required for maximum sodium and strontium activities to appear in intact and in fractured bones at various stages of healing. The line of unit slope is the theoretical locus of points for which the Sr^{85} and Na^{22} times are equal. Each rabbit tested is represented by a point on the charts for fractured and for intact tibiae. The left tibia of each rabbit had been fractured, under anesthesia, prior to the double tracer test. In most cases the ratio of time to Sr maximum ÷ time to Na maximum was greater than one. However, in 4 rabbits whose fractures were very fresh (3 to 5 days), the Sr activity reached a peak before the Na and then declined steadily.

Several investigators have reported that the rates of transcapillary movement of sodium and calcium are very rapid and about equal.[7, 8] The degrees of binding of Ca and of Sr by blood proteins are nearly identical.[16] It is reasonable, therefore, to infer that this difference between the Sr^{85} and Na^{22} records is *not* due to a great disparity in rates of movement through the capillary walls.* If this be true, then the difference may perhaps be ascribed to the well-known "bone seeking" qualities of the alkaline earth strontium. Unfortunately, Na^{22} uptake data cannot be used as a measure solely of blood transport and transcapillary movements. Radiosodium is known to penetrate bone tissue and to be trapped therein by cation exchange processes.[18, 19] As will be shown later, however, the skeletal accumulation of injected Na^{22} is much less than that of Sr^{85}. In any case, the double tracer experiments suggest that the time interval required for Na^{22} activity to reach a peak in bone areas reflects the operation of the processes of vascular transport, transcapillary movement into interstitial fluids, fixation of some of the Na activity by ionic exchange, and return of the

*Recently, however, different transfer rates have been reported for calcium and strontium from plasma to other body fluids, such as peritoneal and cerebrospinal fluid.[17]

remainder to the circulating blood and thence out of the field. Up to this point, an hypothetical Sr^{85} trace would be nearly identical to that for radiosodium. However, *new* bone salt is continually being formed to some extent, even in dense, adult cortical bone, to replace that lost by normal resorption. This deposition of calcium salts traps Sr^{85} ions which have escaped fixation by ionic exchange. Therefore, a much smaller proportion of the arriving Sr^{85} ions are returned to circulation than is true for arriving Na^{22} ions. Blood clearance is therefore faster (Fig. 4) and skeletal retention greater than for Na^{22}. This skeletal accumulation of Sr^{85} continues beyond the time of the Na^{22} maximum and does not level off until the blood concentration of Sr is depleted to a very low level.

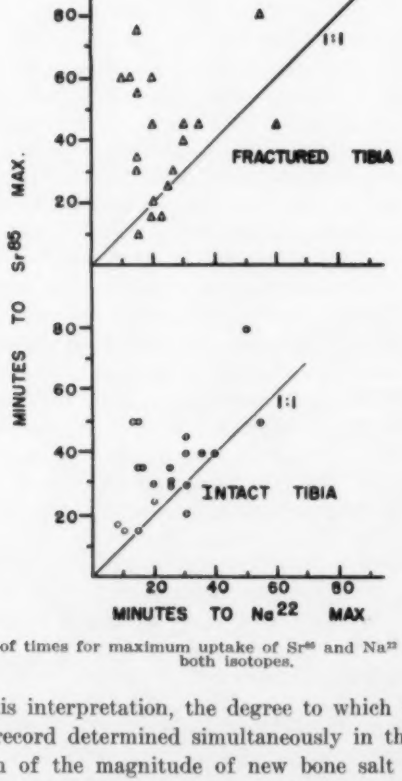

Fig 6—Comparison of times for maximum uptake of Sr^{85} and Na^{22} in bones of rabbits receiving both isotopes.

According to this interpretation, the degree to which the Sr^{85} record deviates from the Na^{24} record determined simultaneously in the same tissue area, is a direct indication of the magnitude of new bone salt formation in that area. Such information could be of great importance in the clinical diagnosis of disorders and lesions of the human skeleton.

Retention and Excretion of Radiostrontium.—The retention and excretion of Sr^{89} and Sr^{90} in bones of rabbits and rats has been reported in terms of power

functions of elapsed time.[5, 6] These values were determined by daily collections of excreta and by serial sacrifice of animals to obtain bone specimens for assay. The advantages of an external counting technique for following the daily loss of activity in each living animal are obvious. Furthermore, if each spot is well marked, the daily losses of activity from various anatomically different areas can be compared. For example it was found that Sr^{85} was lost from cancellous bone in the knee area at a faster rate than from cortical bone in the tibial diaphysis. Healing fractures also exhibited faster declines in radioactivity than cortical bone due, no doubt, to progressive resorption of the calcified callus which contained considerable Sr^{85}. It was necessary to use a pulse height analyzer and to replace the counting ratemeter with a scaler in order to cope with the low levels of activity encountered in these long-term studies. Mathematical corrections for radioactive decay were avoided by counting an aliquot of the original solution used for injection and expressing the activity observed in each bone as a percentage of the counts per minute observed in this standard. Counting times were in all cases long enough to allow at least 1,000 counts to be registered.

TABLE I. CONSTANTS FOR EQUATION DESCRIBING RETENTION OF Sr^{85} IN RABBIT BONES
($R = ct^d$ or $\log R = d \log t + \log c$)

RABBIT NO.	INTERVAL†	d_K	d_T	d_F	$d_K \div d_T$	$d_F \div d_T$
5*	8-57	−0.51	−0.37	−0.73	1.3	2.0
6*	8-57	−0.46	−0.50	−0.73	0.9	1.5
7	10-56	−0.55	−0.50		1.1	
8	4-50	−0.45	−0.35		1.3	
9	3-49	−0.31	−0.19		1.6	
113	11-84	−0.96	−0.40		2.4	
114†	10-90		−0.51	−1.14		2.2
139‡	10-36	−0.97	−0.89		1.1	

Key to abbreviations: R = observed activity; t = days elapsed since injection; c and d are constants. Interval = period during which bones were monitored, in days postinjection. d_K = slope of regression line for knee data; d_T for tibia data; d_F for fracture callus.

*These rabbits were given Sr^{85} when fracture of left tibia was 26 days old.

†Injected with Sr^{85} when left tibia fracture was 10 days old.

‡Na^{22} injected instead of Sr^{85} for comparison of retentions.

When the logarithms of the observed activities were plotted against the logarithms of the elapsed times, a straight regression line could be fitted to the experimental points by the method of least squares. Table I presents the slopes of these regression lines for several animals. The slope for the knee or fracture callus was steeper (i.e., greater rate of decline of activity) than that for the tibia midshaft, indicating greater metabolic activity and bone salt turnover in the first-mentioned areas.

Fig. 7 shows the retention of Sr^{85} in normal rabbit.

Fig. 8 shows the retention of Sr^{85} in a rabbit, injected with Sr^{85} 10 days after a fracture of the left tibia.

Fig. 9 shows the retention of Na^{22} in a normal rabbit.

Excreta from each of 5 rabbits injected with Sr^{85} were collected over a period of nearly two months. Here again the trends could be described very

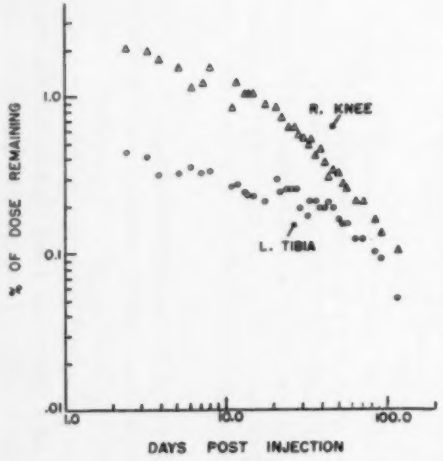

Fig. 7.

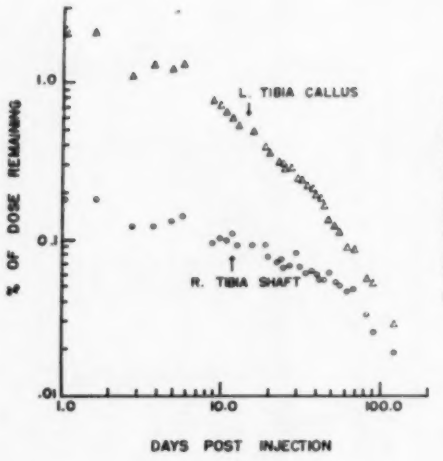

Fig. 8.

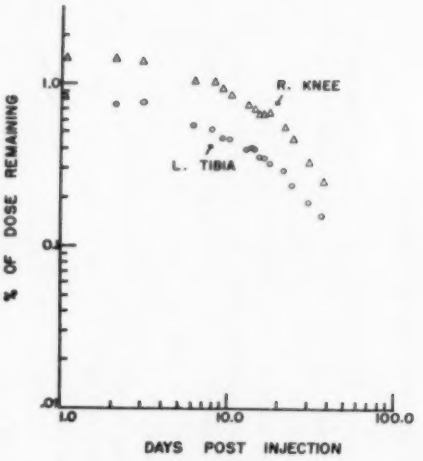

Fig. 9.

Fig. 7.—Retention of Sr^{85} in areas of the knee and tibia midshaft of a normal rabbit. The per cent of dose remaining in each area was estimated by comparing the observed counts per minute to the counts per minute from an aliquot of the solution used for injection, counted on the same day. Because of differences in geometry and scattering, these estimates are crude, but the trends are unmistakable.

Fig. 8.—Retention of Sr^{85} in area of a tibial fracture and in the midshaft of the opposite intact tibia. The radioisotope was injected 10 days after the fracture was made.

Fig. 9.—Retention of Na^{22} in areas of the knee and tibia midshaft of a normal rabbit.

conveniently by plotting the data on log-log coordinate paper, constructing a regression line, and computing its slope. Table II presents the constants for these regression lines.

TABLE II. EQUATION DESCRIBING Sr^{85} EXCRETION IN 5 RABBITS
($E = at^b$)

b	a
−1.8	40
−1.8	45
−1.8	30
−2.1	64
−2.5	324 (subcut.)

Key to abbreviations: E = per cent of dose excreted per day at t days after injection; t = days postinjection; b = constant = slope of regression line on a plot of log E versus log t; a = constant = per cent of dose excreted when t = 1.

The value *b* represents the slope and the value *a* is approximately the percentage of the dose excreted during the first day, obtained by extrapolation back to t = 1. An obviously erroneous value was obtained for *a* in the last animal. In this instance the early data were anomalous because the injection was purposely subcutaneous rather than intravenous.

It should be mentioned that during the first hour after injection, most of the Sr^{85} activity-time records from bone areas showed no decline after reaching the maximum value, except in the case of fractures 3 to 5 days old. However, in 8 experiments in which recording was continued for as long as 2 to 3 hours, slight but steady declines did appear after 1 to 2 hours. These were ascribed to the loss of Sr^{85} from the area by exchange and resorption processes, augmented by continued excretion by the kidneys.[3] In two rabbits whose kidneys had been removed surgically the initial Sr^{85} records for bone appeared normal during the first hour, but no decline was seen during the next 2 hours.

Behavior of Other Radioisotopes.—

Ba^{140}-La^{140}: Although possessing many advantages, Sr^{85} is quite expensive and gamma-emitting Ca^{47} is not available. Ba^{140}, another bone seeking alkaline earth, is quite cheap, however, since it is a waste product of atomic fission. It is obtained in the form of acidic solutions which are in decay equilibrium with La^{140}, its radioactive daughter.

Ba^{140} and La^{140} were separated readily by conventional radiochemical means.[20] Relative purity was checked by utilizing a pulse height analyzer to obtain the characteristic gamma spectrum of each preparation. Activity-time records from bone areas following injections of Ba^{140} were quite similar to those of Sr^{85}, but quite different from those of La^{140}. Peak activities for La^{140} were reached in times ranging from 3 minutes for cortical bone to 10 minutes for knee and fracture sites. Traces were similar to those for Cr^{51}-tagged red cells, but, unlike the red cell records, La^{140} in several cases tended to leave the field slowly after reaching its maximum. If pulse height analyzers are available, there is no need for chemical separation of the Ba^{140} from the La^{140}. Fig. 10 illustrates their comparative behavior in a knee area. A single detector probe was aligned over the knee, its output being split and fed to two separate analyzer-ratemeter-recorder combinations. Both analyzers were adjusted to record Sr^{85}

gammas and this radioisotope was injected. The usual ascending trace for a metabolically active area was obtained on both records. The height of the lower trace is greater than that of the upper only because the range scale for that particular ratemeter was slightly less than for the other ratemeter. After about 40 minutes the window of one analyzer was set to bracket the amplified photovoltages resulting from Ba^{140} scintillations and the other was set for La^{140}. A solution containing the carrier-free equilibrium mixture of Ba^{140}-La^{140} was then injected. The La^{140} maximum was reached within 7 minutes while Ba^{140} activity in the same area was still increasing, like Sr^{85}, at the end of 25 minutes when the experiment was terminated. This finding is in agreement with many published observations that the mode of bone fixation of rare earth elements is quite different from that for alkaline earth elements.[21]

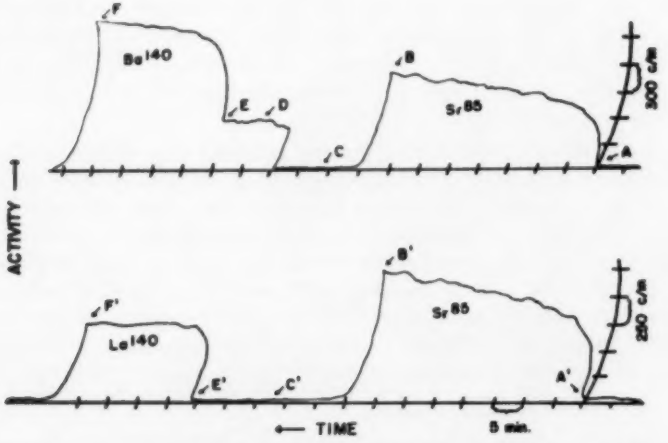

Fig. 10.—Recordings of uptake of Sr^{85}, Ba^{140}, and La^{140} in right knee area of a normal rabbit. Two separate pulse height analyzer-ratemeter-recorder circuits were connected in parallel to the output of a single scintillation detector. Both analyzers were adjusted to record Sr^{85} gammas, and this isotope was injected at the time marked by A and A^1 on the two charts. At time B, the analyzer of this circuit was reset to record Ba^{140}, and, at B^1, the other analyzer was reset to record La^{140}. At C and C^1 the chart speeds were increased so that the records would be conveniently separated. D indicates the portion of the activity from the Sr^{85} injection which was now passing through the window set for Ba^{140}. No Sr^{85} activity was detectable through the La^{140} window because of its much higher setting. E and E^1 mark the injection of a solution containing Ba^{140} in decay equilibrium with its daughter, La^{140}. To keep the Ba^{140} trace on the chart, the ratemeter range scale was immediately raised to 1,000 c.p.m. per chart division. Therefore, the actual counting rate observed for Ba^{140} at F was about 5,500 c.p.m.

Radioiodinated compounds: Fig. 11 shows the build-up of iodide ion I^{131}, administered as sodium iodide solution, in a tibia shaft and in the neck area encompassing the thyroid gland of a rabbit. The blood clearance curve from another experiment is also shown. The initial phases of iodine uptake resemble Na^+ behavior rather than Sr^{++}. Reduction of blood concentrations to low levels was much faster for I^{131} than for Na^{22}, however.

Radioiodinated human serum albumin (RISA) also was tested in 9 animals. Fig. 12 depicts the records of RISA and Sr^{85} pick-up in a 10-day-old tibial fracture and in the intact contralateral tibia of the same rabbit. Both upper and lower records were obtained simultaneously by using a separate

detector-recorder assembly for each leg. It is noteworthy that this protein, which is foreign to the rabbit body, appears to accumulate in the healing fracture, although not nearly so dramatically as Sr^{85}. It is possible, however, that this rise is due to accumulation of iodine ion split off from the foreign protein molecule rather than fixation of the albumin in the area. Radioiodinated rabbit albumin will be prepared in the near future and compared with RISA on a similar experiment. For the present, these preliminary results with RISA

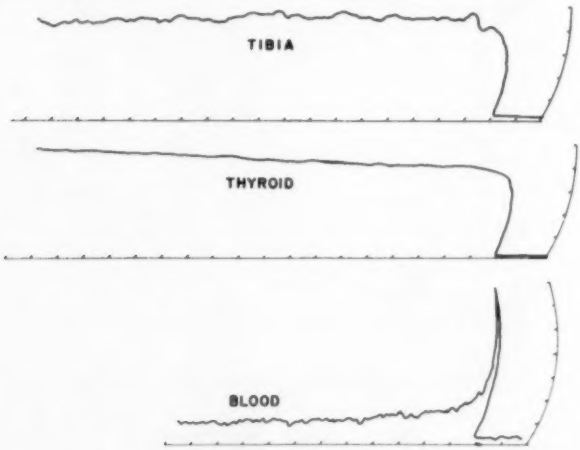

Fig. 11.—Behavior of iodide ion (Na I^{131}) in normal rabbit.

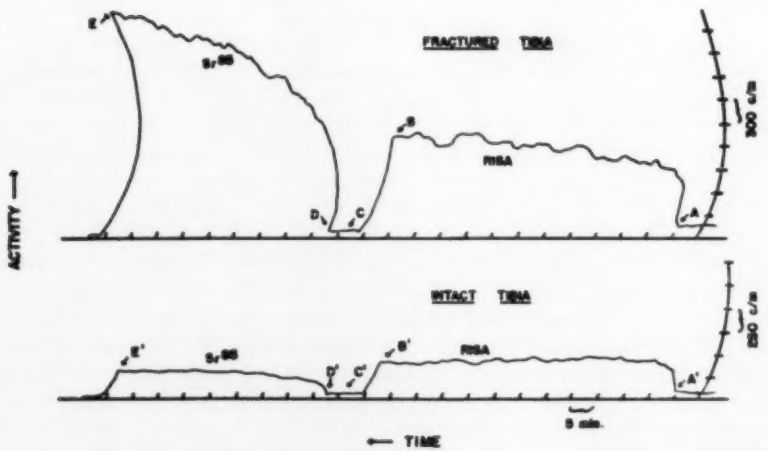

Fig. 12.—Recordings of I^{131}-labeled human serum albumin and Sr^{85} in fractured and intact tibiae of a rabbit. The fracture was 10 days old. One complete detector-analyzer-ratemeter-recorder combination was aligned over the fracture site. Another complete combination was aligned over the midshaft of the opposite tibia. Both analyzers were set to record I^{131} gammas and RISA was injected at the time marked by A and A^1. At B and B^1 both analyzers were reset to record only Sr^{85} gammas. C and C^1 show the background counting rates at these settings. Sr^{85} was then injected at the time marked by D and D^1. Recording was terminated at E and E^1.

suggest that injection of this material just prior to or along with Sr^{85} would make it possible to evaluate the contribution of vascularity to the total picture of Sr^{85} uptake. Indeed RISA has been so used for estimating vascularity in the radioiodinated rose bengal hepatogram for human liver function.[22]

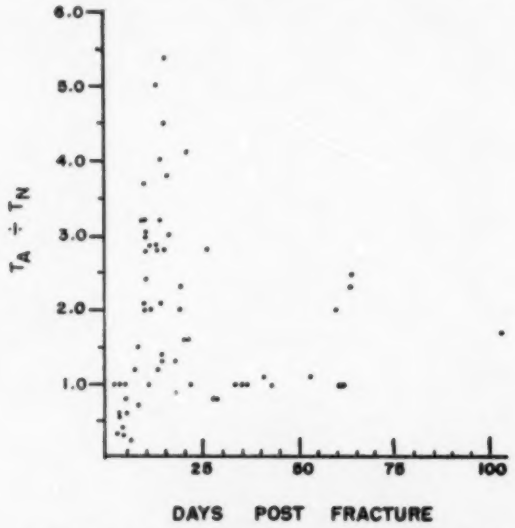

Fig. 13.—Comparison of times for maximum Sr^{85} uptake in fractured and normal bones of rabbits. $T_A \div T_N$ is the ratio (Time to max. in fractured tibia) ÷ (Time to max. in opposite intact tibia).

Radiostrontium in healing fractures: Dynamic studies with Sr^{85} and scintillation counter techniques can provide information on the progress of healing in fractures without harm to the subject. By using two detector-recorder sets, the time required for attainment of maximum activity in the abnormal area can be compared to that required in an equivalent, undamaged area of the opposite, intact limb. Such a comparison discloses the current vigor of repair. In Fig. 13, the ratio of time to maximum activity in fractured (T_A) and in intact (T_N) areas was plotted against the age of fracture for a large number of rabbits. Many of the animals were tested more than once. When the fracture was very fresh (3 to 6 days) the T_A/T_N ratio was less than one. During the most active period of formation and calcification of fracture callus (10 to 22 days) the T_A/T_N ratios became very high. Soon thereafter they dropped back toward unity as the callus sites became adequately calcified. One may infer that the magnitude of the T_A/T_N ratio is a measure of osteoblastic activity in the area of interest.

From the same recorder tracings, additional information may be derived by comparing the maximum *amounts* of radioactivity in each area, i.e., the height of the traces. The detector systems must be adjusted beforehand to equal sensitivity, and the geometries of observation should be made as similar as possible. The data in Fig. 14 show that more Sr^{85} is accumulated in the injured area

than in the intact area at all times postfracture. However, the greatest disparity in uptake occurs between the tenth and twenty-fifth day. This trend is in good accord with Fig. 13 and with observations on healing fractures using Ca^{45} and S^{35}.[23] The increased vascularity of the fracture area is also clearly demonstrated by the data for Na^{22} in Fig. 14. However, it is evident that Na^{22} does not give as useful a picture of the healing process in bone as does Sr^{85}.

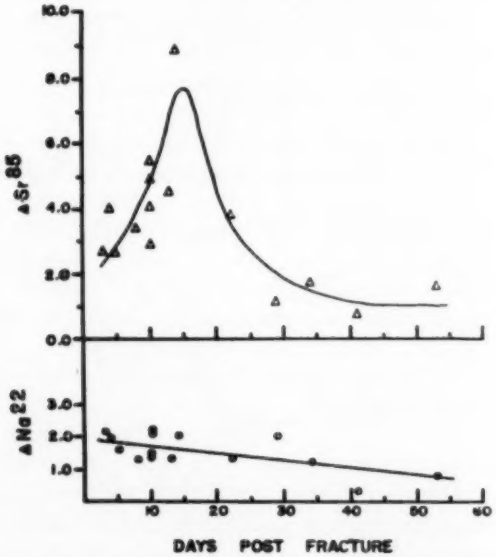

Fig. 14.—Excess strontium and sodium "space" in healing fractures. Both Sr^{85} and Na^{22} were injected into each of this series of rabbits at various time intervals after fracture of the left tibia. A complete detector-analyzer-ratemeter-recorder combination was aligned over the fracture site and another over the opposite, unharmed leg. Both analyzers were set to record 0.51 mev. gamma. Na^{22} was injected, followed by Sr^{85} after the traces had reached plateaus.

$$\text{Excess "space"} = \Delta : \Delta = \frac{\text{(c.p.m. in fractured leg)} - \text{(c.p.m. in intact leg)}}{\text{(c.p.m. in intact leg)}}.$$

The practical value of these external gamma scintillation counting techniques for estimating current metabolic status of a bone area is demonstrated in Fig. 15. A rabbit was injected with Sr^{85} three days after sustaining a fracture of the left tibia. The usual pattern of rapid uptake in the hyperemic area, followed by a slow but steady loss, was obtained. Upon retesting at 16 days, the Sr^{85} record showed that very active calcification was underway in the callus, which was well formed by that time. A roentgenogram showed moderate amounts of new mineral in the area. Another roentgenogram at 60 days showed heavy mineral deposits all around the fracture, which had not been splinted. Were these heavy deposits being augmented by daily accretion of still more new bone salt? A third injection of Sr^{85} was given. The record was interpreted as showing that somewhat more calcium was being deposited than was normal for an undamaged tibia shaft, but not enough to increase greatly the total amount of "surplus" mineral. Indeed, had the area then been counted daily over a

period of a week or so, it is likely that an abnormally rapid *loss* of activity would have been noted. Such additional information would have indicated that the surplus mineral deposits in the callus were not static, but were being resorbed rapidly. The deposition of Sr^{85} during the first hour or so was somewhat greater than normal because of enhanced vascularity and remodeling activities, which involve deposition of mineral at some spots and dissolution at others. However, the ensuing rapid loss of Sr^{85} (see Fig. 8 for example) implies not only a very rapid turnover and replacement of these Sr^{85} ions, but also a net loss as the resorption of mineral outstrips its accretion in the callus.

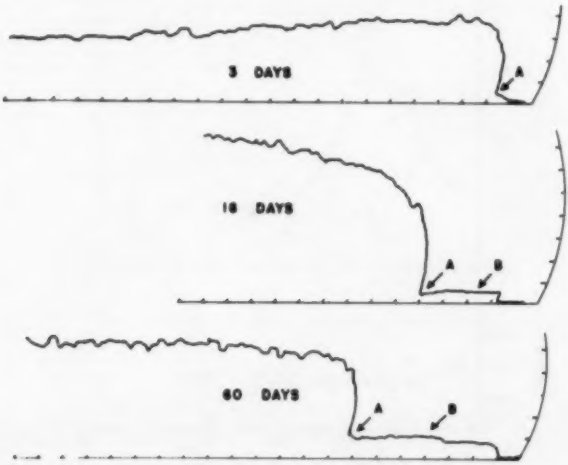

Fig. 15.—Sr^{85} tests of metabolism in healing fracture of a rabbit's tibia.

It must be noted that Sr^{89}, a beta emitter, was used as a tool in studies on bone fracture healing as early as 1945.[24, 25] Also a recent report has appeared, in which the dynamics of bone healing in dogs was studied by repeated daily counts of Sr^{85} activity.[26] Laszlo and his group, however, were the first to use Sr^{85} in human beings.[27] Their carefully controlled, metabolic balance studies have provided basic information on the comparative retention and excretion of strontium and calcium by the human body; others are pursuing similar lines of investigation.[4, 28] It is apparent that sensitive methods are now at hand to examine many different metabolic disorders and lesions peculiar to the human skeleton.* For example, it may be possible to determine easily and quickly whether a localized lesion is in an osteolytic or in an osteoblastic phase. Clinical application of these radioisotope techniques to diagnostic problems such as Paget's disease and to evaluation of therapy of osteoporosis are proceeding under the auspices of the University of California School of Medicine, Los Angeles.

The experiments reported here demonstrate the value of recording and analyzing the changes in radioactivity in selected body areas *during the first*

*G. C. H. Bauer of Malmo, Sweden, recently presented the results of body surface counting of 9 patients who had received both Sr^{85} and Ca^{47}. The uptake of both isotopes in pathologic skeletal lesions was several fold that in normal, unaffected areas.[29]

hour after injection of bone-seeking radioisotopes, as well as at longer intervals thereafter. It is proposed that these time versus radioactivity graphic records be termed "osteograms" (e.g., Sr^{85} osteogram, RISA osteogram). This conforms with the terminology already applied to tracer tests of liver and kidney function, namely, the radioisotope hepatogram and the radioisotope renogram.[22, 30]

SUMMARY

1. Methods were devised for continuously recording variations of radioactivity in bone areas with time, following injection of bone-seeking radioisotopes. These graphic records were named radioisotope osteograms.

2. By using gamma scintillation detectors and pulse height analyzers it was possible to record simultaneously on separate charts the fates of two different radioisotopes following injection of a mixture.

3. These techniques were used in rabbits to compare the clearance from blood and the accumulations in bone of Sr^{85}, Na^{22}, and I^{131}.

4. Double tracer experiments with Sr^{85}, Cr^{51}-tagged erythrocytes and I^{131}-labeled serum albumin elucidated certain kinetic aspects of calcium metabolism, such as vascular transport, transcapillary movements, ionic exchange, and bone salt accretion.

5. Osteograms obtained after injection of Ba^{140}-La^{140}, and Sr^{85} mixtures showed that the behavior of Ba^{140} is quite similar to that of Sr^{85}, but radically different from that of La^{140}.

6. Quantitative comparisons of long-term retentions of Sr^{85} and Na^{22} in various types of bone tissue were made.

7. It was shown that information on the current metabolic status of a fracture can readily be obtained at any point during the course of healing. Osteograms showing the uptakes of Sr^{85} and Na^{24}- or I^{131}-labeled serum albumin *during the first hour* after their injection, followed by daily monitoring of the bone areas, provide the necessary data.

8. Extension of the techniques to the study and diagnosis of metabolic disorders and lesions of the human skeleton is discussed.

Valuable technical assistance in handling animals and analyses was contributed at various times by Mrs. M. Hepler, Miss B. Brooks, Mrs. P. Lorick, Miss L. Petriello, Mr. F. Scholtz, and Mr. P. Noyes. The many stimulating discussions with Drs. G. V. Taplin, M. R. Urist, B. Cassen, and O. Meredith are also gratefully acknowledged.

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EFFECTS OF HEPARIN ON BODY TEMPERATURE AND PLASMA LIPIDS FOLLOWING INTRAVENOUS ADMINISTRATION OF FAT EMULSION IN MAN

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INTRAVENOUS injection of heparin is capable of clearing alimentary lipemia in vivo.[1-3] Bragdon and Havel[4] and French and associates[5] have shown that administration of heparin to rats increased the rate of removal of the intravenously injected lipoproteins and fatty chyle, respectively. Grossman and Strub,[6] using rats, and Meng and Youmans,[7] using dogs, found that heparin injected simultaneously with synthetic fat emulsion also accelerated the disappearance of the infused fat from the blood circulation. This increase in rate of removal of plasma lipids following heparin administration is presumably due to the production of lipemia clearing factor which is lipolytic in nature. It is well established that plasma obtained after heparin injection hydrolyzes the synthetic triglyceride emulsion or triglyceride component of lipemia plasma in vitro.[8, 9] The present study was undertaken to investigate the effects of heparin administered simultaneously with intravenous infusion of fat emulsion on plasma unesterified fatty acids and on body temperature in man.

MATERIALS AND METHODS

Thirty-three patients† were selected on the surgical wards at the Veterans Administration Hospital in Nashville. These patients can be divided into 3 groups. (1) Twenty-five were postoperative patients with various pathologic conditions, such as carcinoma of the lung, duodenal ulcer, hernia, appendicitis, etc. Infusions of fat emulsion were given usually from 2 to 10 days after the operation. (2) Three patients were in the convalescent state after long bone fractures or burns. (3) Five patients were given fat infusion before operations. The age of these patients ranged from 22 to 68 years.

Plasma unesterified fatty acid and plasma total lipids were determined in 12 of the 33 patients whose ages ranged from 23 to 39 years. Of these 12 patients, 5 were postoperative cases, such as hemorrhoidectomy and other minor surgeries; 4 preoperative cases; and 3 with traumatic ulcer or skin graft of lower extremities. None of these subjects were severely ill and they were afebrile. Eight of these 12 patients required nutritional therapy, but the other 4 were in fairly good nutritional condition. None of the 33 patients was cachectic.

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Six hundred milliliters of Lipomul*, a 15 per cent cottonseed oil emulsion, was given by intravenous drip. The rate of infusion was carefully regulated and all infusions were completed in 3½ to 4 hours. Each patient received two infusions of fat emulsion—the first without heparin and the second with heparin.† The heparin dosage was 100 mg. given intravenously 3 to 5 minutes prior to, and 50 mg. given immediately after, the completion of fat infusion. The second infusion of fat emulsion was given within 5 days after the first in all cases, although usually within the next two days.

Oral temperature was taken before, at mid-point during, at the end of, and hourly for 4 hours after completion of fat infusion. Blood samples were withdrawn for plasma unesterified fatty acids and total lipids before and at intervals during and after completion of fat infusion. The modified method of Borgstrom[10] was used for the determination of plasma unesterified fatty acids. The plasma total lipids were determined by gravimetric method of Street[11] with a slight modification.

RESULTS

The effects of heparin on changes in plasma unesterified fatty acids and total lipids following intravenous infusion of Lipomul are presented in Table I. It can be noted that the plasma unesterified fatty acids were increased whether or not heparin was given. However, the increase observed at the end of infusion was significantly greater with heparin than without heparin. The mean difference of plasma unesterified fatty acids between fat infusions with and without heparin determined 3 hours after completion of infusion was not statistically significant.

TABLE I. EFFECTS OF HEPARIN ON CHANGES OF PLASMA UNESTERIFIED FATTY ACIDS AND TOTAL LIPIDS FOLLOWING INTRAVENOUS INFUSION OF FAT EMULSION

ANALYSIS	PLASMA UNESTERIFIED FATTY ACIDS (mEq./L.)			PLASMA TOTAL LIPIDS (MG. PER CENT)				
					1 HR.		AFTER INFUSION	
TIME	BEFORE INFUSION	END OF INFUSION	3 HR. AFTER INFUSION	BEFORE INFUSION	DURING INFUSION	END OF INFUSION	1 HR.	3 HR.
Mean								
Nonheparin	0.493	1.464	0.162	681	1,199	1,963	1,579	1,492
Heparin	0.543	4.136	0.812	722	1,043	1,715	1,254	922
Mean difference* ± S.E.	0	2.607	0.188	0	172	329	396	587
P		<0.001	>0.1		>0.1	= 0.009	= 0.004	<0.001

*Mean difference between heparinized and nonheparinized groups. For plasma unesterified fatty acids: (mean difference from preinfusion level of heparinized group)—(mean difference from preinfusion level of nonheparinized group. For plasma total lipids: (mean difference from preinfusion level of nonheparinized group)—(mean difference from preinfusion level of heparinized group).

As was expected, the plasma total lipids were elevated as the results of fat infusion. However, the increase in plasma total lipids was not as great and returned to the preinfusion level sooner when heparin was given simultaneously with fat. The mean differences in plasma total lipids between fat

*Lot Nos. 11612-22, -33, -43, -50, and -61 were supplied by The Upjohn Company, Kalamazoo, Mich., through the courtesy of Dr. E. A. Hawk. The emulsion contains 15 per cent cottonseed oil and 4 per cent glucose.

†Heparin sodium, 10 mg. per milliliter and 100 U.S.P. units per milligram, was kindly furnished by Dr. E. A. Hawk, The Upjohn Company, Kalamazoo, Mich.

infusions with and without heparin were 329, 396, and 587 mg. per cent, respectively, at the end of, and 1 and 3 hours after, infusion. These differences are statistically significant (Table I).

Fig. 1 shows the changes in body temperature during and following fat infusion both with and without heparin treatment in 33 patients. The results are expressed in terms of mean temperature difference from preinfusion levels.

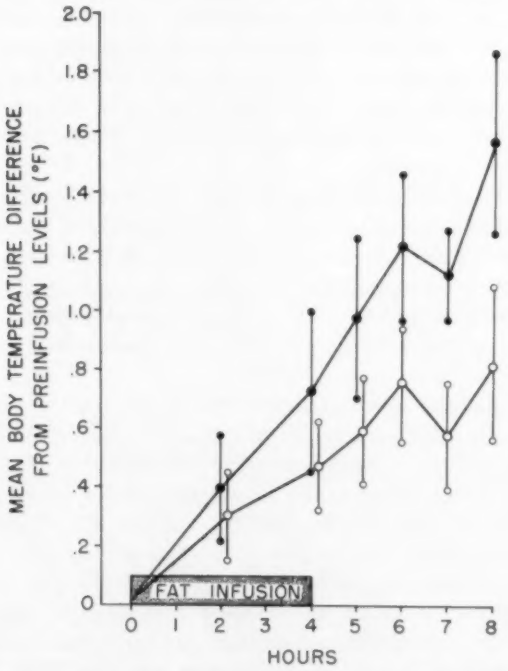

Fig. 1.—Mean changes in body temperature following intravenous infusion of fat emulsion. ●——● = Nonheparinized group; ○——○ = Heparinized group. Vertical lines represent the standard errors of the means.

It can be seen that body temperature progressively increased following intravenous infusion of fat emulsion, whether or not heparin was administered. The mean elevation in temperature measured at all points during and after fat infusion was significantly higher ($p = 0.05$ or less) than the preinfusion level, except that at the mid-point during infusion in the group receiving heparin.

The changes in body temperature following intravenous infusion of fat emulsion in 12 patients whose plasma lipids were determined were identical with those observed in the entire group of 33 patients.

It should be pointed out that of the 66 infusions in 33 patients, 8 produced thermogenic response of more than 2° F. (2° F., 5 infusions; 3° F., 2 infusions;

and 4° F., 1 infusion); the incidence is, therefore, 12.1 per cent. Of the 8 infusions which produced fever, 7 received no heparin (21 per cent) and 1 received heparin (3 per cent). No other type of untoward reaction was observed during or after infusion with or without heparin.

DISCUSSION

We have confirmed in previous studies[6, 7] in animals that administration of heparin increases the rate of removal of the intravenously infused synthetic fat emulsion from the circulating blood. Heparin administration presumably leads to the production of lipemia clearing factor which hydrolyzes triglycerides. The observations in the present study of a marked increase in plasma unesterified fatty acids in patients receiving fat emulsion plus heparin would support such a conclusion.

It is apparent from the data presented that intravenous infusion of fat emulsion produces low-grade elevation of body temperature. Since only 5 per cent of the 66 infusions were associated with an increase in temperature of more than 3° F. and no other undesirable reaction was observed, it seems that this preparation was fairly well tolerated, at least in short-term studies. However, the mechanism of the slight elevation of body temperature remains to be explored.

It was postulated that accumulation of unesterified fatty acids in the blood stream might in itself produce fever, or cause hemolysis which results in an increase in plasma-free hemoglobin which subsequently produces elevation of body temperature. Thus, if the above postulations were correct, administration of heparin simultaneously with fat emulsion might aggravate the thermogenic response. However, the present work does not seem to support such a hypothesis, although the increase in plasma unesterified fatty acids was much greater in patients receiving fat emulsion with heparin than in those without it. In fact heparin might have reduced the incidence of thermogenic reaction. Since in most of the 33 patients heparin was given with the second infusion of fat emulsion, it is possible that the lesser temperature rise might have been due to the development of "tolerance" to fat.

It is of interest to point out that Mueller[12] and Becker and Buxbaum[14] have demonstrated a direct correlation between rapid clearing of the intravenously infused fat from the blood stream and the occurrence of fever in patients. Lambert and associates[13] have shown that the rate of clearance of the infused fat is delayed in rabbits receiving emulsions incorporated with Triton A-20; the incidence of fever is also eliminated in these animals with slow clearance of fat. However, Becker and Grossman[15] and Moeller and associates[16] have reported that there is no apparent relationship between the rate of infusion and the incidence of thermogenic reaction in rabbit and in man, respectively. The findings presented here indicate that heparin did increase the rate of removal or clearance of the infused fat, yet the elevation

of body temperature in patients receiving heparin was less than that in those receiving no heparin. Thus, it seems apparent that the rate of removal of the infused fat per se is probably not related with the incidence of fever.

SUMMARY

The effects of heparin on plasma unesterified fatty acids, plasma total lipids, and body temperature following intravenous infusion of synthetic fat emulsion in patients have been studied.

Administration of heparin increased the rate of removal of the infused fat emulsion from the blood stream. This increased removal of the infused fat is, at least in part, due to increased lipolysis as indicated by the greater increase in plasma unesterified fatty acids in patients receiving fat emulsions with heparin than in those without it.

Low-grade elevation of body temperature was observed following intravenous infusion of fat emulsion. Administration of heparin simultaneously with fat emulsion did not seem to aggravate the thermogenic response.

We wish to express our appreciation to Dr. Margaret P. Martin and Mr. Ben Couch, Department of Preventive Medicine, Vanderbilt University School of Medicine, for statistical analysis of data.

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ON THE COMPOSITION OF CHYLE CHYLOMICRONS

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GAGE and Fish[1] coined the term "chylomicron" to identify those particles which exhibited brownian movement in dark-field preparations of plasma and which had a diameter of at least 0.5 micron. They demonstrated that these particles contained lipid, and that they increased in number in both the chyle and plasma following the ingestion of fat but not following the ingestion of protein or carbohydrate. We now know, largely from the work of the Donner group,[2] that there are also, at least in some lipemic human plasmas, in addition to the particles described by Gage and Fish, a spectrum of lipoprotein micelles, many too small to be seen as entities in dark-field examination and yet large enough to scatter light. According to Lindgren and associates,[3] lipoproteins with Sf values greater than 400 scatter light. With present methods it is impossible to subfractionate these particles into homogeneous classes. In this communication the term chylomicron wil be used to refer to all the light-scattering lipid particles, although in actuality the preparations used contained predominately the larger particles.

Although there is general agreement among investigators that chylomicrons consist primarily of triglycerides and that when separated from blood they appear to contain 1 to 2 per cent protein, there is no such agreement regarding the protein content of chylomicrons separated from chyle. Chylomicrons from blood become disrupted when repeatedly washed through saline, a phenomenon first reported by Weld.[4] Such is not true of chylomicrons separated from chyle. Laurell[5] recovered chylomicrons from rat chyle by flocculation with toluidine blue and reported a protein content of about 2 per cent. The same amount was found in dog chyle chylomicrons by Havel and Fredrickson[6] after centrifugal separation. They did not, however, wash their preparations. Albrink and associates[7] reported that in both human and dog chyle chylomicrons the protein content was too low to measure with accuracy. Robinson,[8] who has made extensive studies of chyle chylomicrons in the rat, concluded that the small amount of protein found on first isolation could be completely washed away by repeated centrifugations through physiologic saline. In view of these conflicting data it seemed appropriate to report the experiences of this laboratory.

METHODS

Polyethylene cannulas were inserted in the cysternae chyli of rats and the animals were placed in restraining cages. They were offered physiologic saline to drink and a diet

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of ground pellets saturated with olive oil in order to increase the yield of chylomicrons. For the separation of chylomicrons, the whole chyle was layered under physiologic saline in plastic tubes of the 40 rotor of the Spinco preparative ultracentrifuge. Centrifugation was performed under different conditions described below. Under our usual conditions the chylomicrons formed a butterlike mass at the top of the tube. They readily re-emulsified in physiologic saline, through which they were washed at least 2 more times. For the determination of lipids, 1 ml. of the washed emulsion was extracted in a final volume of 25 ml. of chloroform-methanol (2:1, v/v). After standing at least 30 minutes at room temperature, 5 ml. of water was added and the 2 phases permitted to separate overnight. Aliquots of the lower phase were taken for determinations of cholesterol,[9] lipid phosphorus,[10] and total lipid.[11] For the determination of protein, 1 ml. of the washed emulsion was extracted in a final volume of 25 ml. of ethanol-acetone (1:1, v/v). On the following day the protein precipitate was centrifuged down and was washed once in ethanol-acetone to free it of entrapped lipid. It was then dissolved in 0.5 ml. of 1 N NaOH, and the amount of protein was determined by the very sensitive method of Lowry and associates.[12] As this method measures only the tyrosine content of the protein, it was compared with the biuret method for rat chylomicron protein, rat serum proteins, and human serum proteins. The concentration of the last was also determined as 6.25 times the Kjeldahl nitrogen. Results of these comparison showed that the tyrosine content of rat chylomicron protein was the same as that of rat serum proteins and that both of these contained only 85 per cent as much tyrosine as do human serum proteins. Suitable corrections have been made.

Carbon[14] radioactivity in protein was determined by the method of Vaughan, Steinberg, and Logan.[13] After extraction in ethanol-acetone, the protein precipitate was washed, dried, and then dissolved in a 1 M solution of Hyamine in methanol. The protein remained in solution after the addition of toluene containing the phosphor 2,5-diphenyloxazole. A Packard Tri-Carb scintillation counter was used. All counts were corrected for self-absorption by recounting after the addition of a known amount of carbon[14].

TABLE I. EFFECT OF DIET ON LIPID COMPOSITION OF CHYLE CHYLOMICRONS

DIET	TOTAL LIPID* (MG.)	CHOLESTEROL (PER CENT)	PHOSPHOLIPID (PER CENT)
Mash + olive oil	198	0.66	6.9
Mash + soy lecithin	201	0.43	6.8
Mash + olive oil + chol.	219	1.9	7.2

*In chylomicrons from 5 ml. of chyle.

RESULTS AND DISCUSSION

1. *Effect of Diet on Lipid Composition of Chylomicrons.*—This was investigated by offering the following diets during 3 consecutive daily periods and analyzing the chylomicrons obtained at the end of each period: (1) purina pellets ground to a mash and saturated with olive oil; (2) mash mixed with soy lecithins; (3) mash saturated with olive oil in which cholesterol was dissolved. The chyle samples were layered under saline and centrifuged for 30 minutes at 100,000 g. The results are presented in Table I, where it can be seen that the feeding of cholesterol led to a threefold increase in the cholesterol content of the chylomicrons. The same effect can be observed in data published by Byers and Friedman.[14] The feeding of large amounts of lecithin, on the other hand, did not raise the concentration of phospholipid in the chylomicrons. This was true despite the fact that the concentration of total lipid in this chyle sample was just as great as after olive oil feeding. This indicates

that practically all of the phospholipids fed were hydrolyzed in the intestine and that their fatty acids appeared as triglycerides in the chylomicrons. The phosphorus-containing moiety was apparently transported via the portal system. These findings confirm those of Byers and Friedman[15] who compared the total chyle content of phospholipids after feeding olive oil with that after feeding olive oil containing cholesterol and phospholipid. The addition of phospholipids to the oil did not increase the total amount of phospholipid in the chyle. Bloom and associates[16] and Blomstrand[17] fed rats either phospholipids labeled with C^{14} in the fatty acids or triglycerides labeled in the fatty acids. They then compared the distribution of the label between the phospholipids and the triglycerides of the whole chyle. After feeding labeled phospholipids a greater percentage of the absorbed label was in chyle phospholipids than after feeding labeled triglyceride. Both concluded that most of the fed phospholipid had been completely hydrolyzed, but that a small amount had apparently been absorbed intact.

2. *The Protein Content of Chyle Chylomicrons.*—Chylomicrons were prepared under the centrifugal conditions suggested by Lindgren, Elliott, and Gofman.[18] Whole chyle was layered under saline and centrifuged for 10 minutes at 9,500 g only. Only that material which had packed out at the top of the tube was recovered. This was re-emulsified in saline and centrifugation repeated 4 times. These centrifugal conditions were not sufficient to pack out all the light-scattering particles, so that many were discarded after each centrifugation. It is impossible to define exactly which classes of chylomicrons were represented in the final material, nor is there any reason to believe that they were homogeneous. This represents the very lowest density chylomicrons and the protein content as a percentage of total weight was 0.2 per cent. Assuming inhomogeneity, it is possible that some of the particles were actually protein-free. Chylomicrons separated under these conditions also contain less phospholipid than do those recovered after 30 minutes at 100,000 g.

Because in the above preparation so much light-scattering material was lost at each centrifugation, the usual procedure adopted was to use the greater centrifugal force described above and to wash the chylomicrons under the same conditions. It must be realized that these are sufficient to cause some of the denser, non-light scattering lipoproteins, should they be present, to reach the top of the tube. That they did not constitute a significant contamination was shown by two methods. Analytical ultracentrifugation showed no lipoproteins of density greater than Sf 400 to be present in detectable amounts. Further, when a cannulated rat was fed palmitic-acid-C^{14} without carrier fat, the specific activity of the lipids recovered after the 30-minute centrifugation was just as great as the specific activity of the lipids recovered after the preliminary 10-minute centrifugation. This indicated again that there was no significant contamination by preformed lipoproteins which might have been present in the chyle as a transudate from blood.

Chylomicrons obtained from rats absorbing olive oil, prepared and washed by 30-minute centrifugations at 100,000 g, contain approximately 0.5 per cent protein.

3. *The Nature of the Lipid-Protein Binding.*—Because Robinson[8] believed he could completely remove the protein from chyle chylomicrons by repeated washing, he concluded that it was not an integral part of the chylomicron structure but was merely adsorbed to the surface when the chylomicrons were first separated from the chyle. Table II presents the results of repeated centrifugal washings through physiologic saline of a sample of rat chylomicrons and a sample of human chylomicrons. The latter were obtained from a patient with chylothorax. The centrifugal conditions were 30 minutes at 100,000 g and it can be seen that consecutive washings did not reduce the protein content of either sample. It is believed that Robinson's failure to find protein was the result of inadequate analytic methods. He extracted the chylomicrons in a two-phase ether-water system and measured the nitrogen in the water phase by the Kjeldahl method. The nitrogen contributed by the phospholipids had to be subtracted.

TABLE II. THE EFFECT OF REPEATED WASHINGS ON THE PROTEIN CONTENT OF CHYLE CHYLOMICRONS

NO. WASHES	PER CENT PROTEIN	
	RAT	HUMAN
2	0.45	
3	0.46	
4	0.45	
5	0.45	0.55
6		0.58
7		0.59

Lindgren[3] has suggested that chylomicrons may consist of a lipid core surrounded by a protein shell. Bull[19] has shown that 1 mg. of protein in a monolayer occupies an area of approximately 0.78 M.^{2}. For a lipid sphere with a diameter of 0.5 μ it can be calculated that sufficient protein to cover the surface would comprise 1.5 per cent of the mass by weight. Chylomicrons from chyle do not contain this much protein. Robinson[8] has shown that chylomicrons are disrupted by exposure to *Clostridium welchii* lecithinase, suggesting that the stability of the chylomicrons in an aqueous medium is at least partially maintained by the phospholipids which they contain.

4. *The Origin of Chylomicron Protein.*—It is well known that the principal route of absorption of amino acids is via the portal vein to the liver. If chylomicron protein were synthesized de novo in the intestinal wall, the feeding of labeled amino acids along with fat to a cannulated rat should result in high specific activities in the chylomicron protein shortly after feeding and this high activity should fall off rapidly as the absorbed label became diluted in the larger amino acid and protein pool of the entire animal. On 3 separate occasions a rat was fed a mixture of labeled amino acids with olive oil. In each instance the specific activity of the chylomicron protein was highest in the first hour. Only the third of these experiments will be reported in detail.

On the day following cannulation a rat that was eating ground pellets saturated with olive oil was fed by stomach tube a mixture of the following amino acids labeled with C^{14}: glycine, leucine, lysine, phenylalanine, serine, and valine. The total radioactivity was approximately 130 μc. Collections of lymph were made hourly for the first 6 hours and thereafter from the sixth to thirteenth and from the thirteenth to twenty-first hours. The flow varied from 2 to 3 ml. per hour. This amount from each of the 8 collections was layered under saline and the chylomicrons were separated and washed as described above.

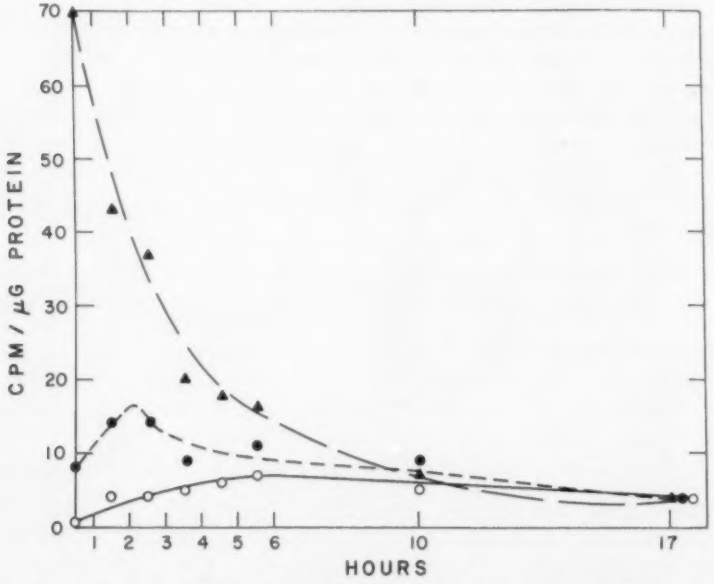

Fig. 1.—The specific activities of chylomicron protein (▲), lipoprotein protein (●), and residual proteins (○) of the chyle after the feeding of C^{14}-labeled amino acids in a rat. In each case the points have been plotted midway through the collection period.

The final preparations were extracted in ethanol-acetone in duplicate. The protein content was determined on one of these, and the radioactivity was determined on the other, after washing the precipitates in additional ethanol-acetone to free them of lipid radioactivity. The infranatant chyle from the first centrifugation was again layered under saline and recentrifuged for 30 minutes to free it of any possible chylomicron contamination. The upper layer from this centrifugation was discarded. The chyle infranate was then brought to a saline density of 1.21 by the addition of an appropriate amount of NaCl and KBr and centrifuged for 25 hours in the 40.3 rotor at approximately 114,000 g. By this means all the lipoproteins, which in the rat are predominantly high-density (alpha-1) lipoproteins, were separated from the residual proteins. Both lipoproteins and residual proteins were exhaustively dialyzed against physiologic saline to free them of excess salt and any free amino acids. The specific radioactivity of

these proteins was also determined. Fig. 1 shows the results expressed as counts per minute per microgram of protein for each fraction plotted against time. It can be seen that the specific activity of the chylomicron protein was at a high maximum during the first hour, and that it far exceeded the specific activity attained at any time by any other fraction. It fell rapidly from its initial high values. The specific activity of the lipoproteins reached a peak during the second and third hours and then slowly declined. The residual proteins attained a maximum activity during the sixth hour and then slowly declined.

To prove the adequacy of these methods in excluding free amino acids and to show the absence of in vitro exchange phenomena between free amino acids and proteins, a mixture of the same labeled amino acids that had been fed was incubated in vitro with 3 ml. of whole nonradioactive chyle. The chylomicrons were separated and washed exactly as in the in vivo experiment. The infranatant material, containing in this instance both lipoproteins and proteins, was also treated exactly as in the in vivo experiment. Both samples showed radioactivity only at background levels.

The rapid appearance of high-specific activity in the chylomicron protein following the feeding of radioactive amino acids suggests, but does not prove, that part at least of the chylomicron protein is synthesized in the intestinal wall.

SUMMARY

Chylomicrons in chyle are not a homogeneous entity but represent a spectrum of lipid-protein particles large enough to scatter light. They consist primarily of triglycerides, but also of phospholipids, cholesterol, and protein. The cholesterol content of chylomicrons can be increased by cholesterol feeding, but the phospholipid content does not increase following the feeding of phospholipids. This is interpreted as indicating that practically all of the fed phospholipids are hydrolyzed in the intestinal lumen and that the phosphorus-containing moiety is absorbed via the portal system.

Chylomicrons of the very lowest density contain 0.2 per cent protein by dry weight. The average protein content of the light-scattering particles which appear in chyle after fat feeding is about 0.5 per cent. The protein content cannot be reduced by repeated washings through physiologic saline, indicating that protein is an integral part of the chylomicron, although it is insufficient in amount to cover the chylomicron surface. Following the feeding of radioactively labeled amino acids, the protein of the chylomicrons shows its highest specific activity in the first hour. Thereafter the specific activity falls rapidly. This suggests, but does not prove, that at least some of the chylomicron protein is synthesized in the intestinal wall.

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EXPERIMENTALLY INDUCED PERMANENT UNILATERAL RENAL DISEASE IN DOGS

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DETAILED investigation of the functional capacity of the contracted kidney in man has been impeded by a number of technical and conceptual obstacles. Of major importance among these have been: (1) the difficulty in establishing the precise relationship between clearance values and specific renal functions in the presence of advanced renal insufficiency; (2) the infrequent opportunity to relate data obtained from the patient with Bright's disease to control observations from the same subject; and (3) the inability to document the specific effects of body fluid abnormalities and adaptive changes in glomerular-tubular balance, evoked by the renal disease, on the function of the persisting nephrons.

One means of circumventing these obstacles would be to develop techniques for inducing chronic renal disease in a single kidney of experimental animals. The presence of one normal kidney and one contracted kidney in the same animal, in which both kidneys may be studied serially and simultaneously, would appear to offer several advantages. The functions of the diseased kidney may be compared with control values (i.e., before induction of the lesion) of the same organ. In addition, data from the experimental kidney may be related to the concurrent values of the contralateral intact kidney throughout the course of the disease. Comparisons of this nature would provide a basis for assessing the validity of the experimental methods used in studying the contracted kidney. Moreover, the fact that both diseased and intact kidneys reside in a common internal environment, receive the same extrarenal stimuli, and are perfused with glomerular filtrate of identical composition enhances the value of the comparison in evaluating the functional capacity of the diseased organ.

The presence of the intact kidney could offer another significant advantage. Regardless of how extensive the disease is in the experimental kidney, the total complement of nephrons of the unaltered kidney would remain intact. Hence,

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the existing (bilateral) nephron population must always exceed 50 per cent of the original number. Because of this, major alterations in the chemical composition of body fluids would be prevented. Moreover, adaptive changes in nephron function, which might be initiated by the more severe diminution of total nephron population occurring in bilateral renal disease, would be minimized. Administration of specific solutes (e.g., urea) either on a short-term or long-term basis would permit the investigation of the influence of body fluid alterations on the function of the contracted kidney; the ultimate removal of the normal kidney (either in a single procedure or by successive partial nephrectomies) could reveal the occurrence and nature of changes in glomerular-tubular balance. Any measureable adaptive changes developing in the *intact* kidney, prior to its removal, would also be discernible by comparison of serial clearance values of this kidney with its control values.

The present report describes observations on the experimental induction of chronic unilateral renal disease in dogs.

METHODS

Experiments were performed on female mongrel dogs weighing 10 to 20 kilograms. A preliminary bladder-splitting surgical procedure was performed in order to provide each kidney with a permanent hemibladder. The method, as described by Desautels,[2] consists of dividing the urethra approximately 2 cm. from the bladder and ligating the distal end. The proximal urethra and bladder are split longitudinally, carefully incising the trigone midway between the ureterovesical orifices. Each hemibladder is then closed over a flanged polyethylene catheter (internal diameter, 3/16 inch) by a continuous inverting suture. The catheters, which are 10 cm. in length, are brought to the exterior through stab wounds in the right and left anterior abdominal wall. Urine drains freely through the plastic tubes and, providing appropriate precautions are taken to prevent obstruction, the animals will remain healthy and free of significant pyelonephritis for indefinite periods.

Induction of Unilateral Renal Disease.—After obtaining control clearance studies to evaluate the functional interrelationships of the two kidneys, unilateral renal disease is induced in the following manner (Fig. 1). Employing sodium pentobarbital anesthesia, a mid-line abdominal incision is made and both kidneys are exposed, examined grossly, and biopsied. One kidney* is mobilized and the renal artery and vein are freed by blunt dissection from the hilus to the aorta and inferior vena cava, respectively. The capsular blood supply is interrupted to prevent collateral circulation during the perfusion procedure. The renal artery is then occluded by a small bulldog clamp, thereby rendering the kidney totally anoxic, and a Mulligan vein clamp is placed on the vena cava at its junction with the renal vein. The Mulligan clamp is closed, and a small incision is made in the vena cava near the entrance of the renal vein. A polyvinyl catheter is passed through the stab wound into the renal vein where it is secured with umbilical tape. A 22 gauge needle is then inserted into the renal artery between the bulldog clamp and the kidney. Care is taken to place the needle proximal to any bifurcation of this artery. After the kidney has been anoxic for 30 minutes, it is perfused over a 10-minute period with 100 ml. of isotonic saline containing from 0.75 to 3.0 Gm. of an aminonucleoside (6-dimethylaminopurine-3-amino-D-ribose).† The perfusing solution is drained to the exterior through the catheter in the renal vein and recirculation is thereby prevented. After completion of the perfusion, the catheter in the renal vein is removed and the incision in the vena cava closed with 5-0 arterial silk. The

*The right kidney has been found to be technically more satisfactory because of the greater ease of catheterizing the right renal vein through the inferior vena cava.

†The aminonucleoside was generously supplied by the Lederle Laboratories Division, Pearl River, N. Y.

Mulligan and the bulldog clamps are removed, and renal blood flow is re-established. The total period of renal anoxia is approximately 60 minutes. When blood loss is excessive, transfusion is performed prior to completion of the procedure. With increasing proficiency, however, blood loss has been minimal and transfusion avoided.

Clearance studies were performed with the animals in the unanesthetized state and the standing position, loosely supported by an infra-abdominal sling. Details of the clearance procedures and analytic techniques have been described previously.[3] Bilateral renal biopsies were obtained surgically in the control state and serially after induction of the lesion. Blood pressures were measured on a Sanborn direct recording apparatus, using a 20 gauge needle in the femoral artery.*

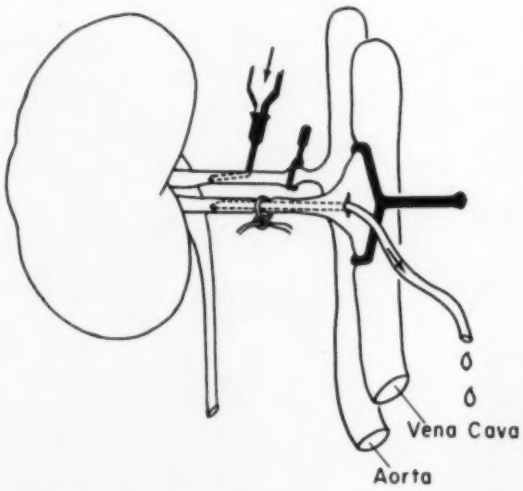

Fig. 1.—Induction of unilateral renal disease. See text for description.

RESULTS

Induction of unilateral renal disease has been successfully accomplished in 8 dogs. A moderate to severe decrease in function of the experimental kidney has been noted in each of these animals. Serial renal function studies on 3 animals with marked lesions are shown in Fig. 2. The depression of renal function has been maximal in the period immediately following the experimental procedure. This presumably reflects, in part, a reversible component secondary to the prolonged anoxia sustained during the renal perfusion. A limited increase in function has typically occurred over a period of 6 to 8 weeks, following which clearance values have stabilized at levels appreciably below the control values. In one of the 8 dogs, a delayed decrease in function of the experimental kidney occurred. In two others, a secondary increase in glomerular filtration rate and renal plasma flow was noted after several months. The latter, however, was accompanied by proportional changes in the intact kidneys and cannot be ascribed to reversal of the lesion in the diseased kidney.

*We are indebted to Dr. Philip Comens, Hypertension Division, for obtaining these measurements.

Because subcutaneous injections of the aminonucleoside employed in the present studies have been noted to induce a nephrotic syndrome in rats,[4, 5] urinary protein excretion and plasma protein values were determined serially. Marked proteinuria from the experimental kidney (i.e., 3 to 4+ qualitative reaction with Esbach's reagent) was noted only during the first few days following induction of the lesion and thereafter only trace to 1+ concentrations occurred. The urine from the intact kidneys was unaffected by the contralateral perfusion procedure. Determinations of plasma total protein and albumin levels after induction of the lesion revealed no consistent change from the control values in any of the animals.

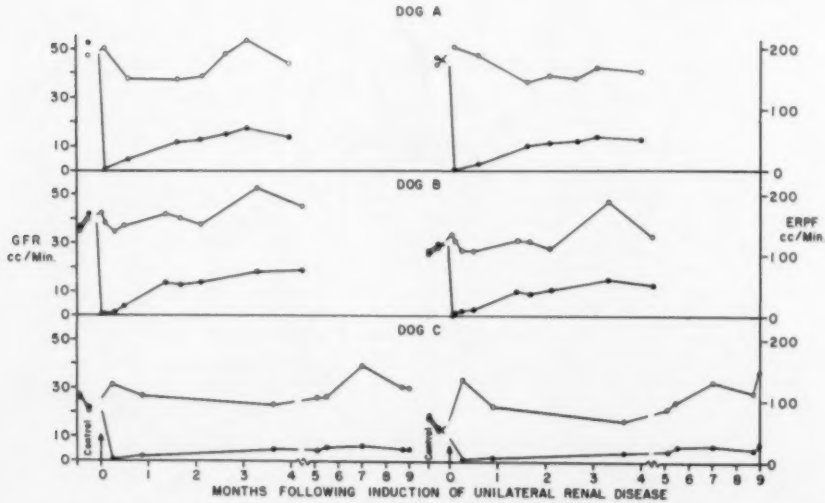

Fig. 2.—Serial bilateral renal function studies following induction of unilateral renal disease. GFR = glomerular filtration rate; ERPF = effective renal plasma flow. Open circles = intact kidney; closed circles = experimental kidney; arrows indicate time of induction of lesion.

Anatomically, the experimental kidneys showing significant functional impairment have developed marked alterations. Decrease in renal mass has occurred over a 2- to 4-month period such that the kidneys have become contracted often to less than 25 per cent of their original size. The surface of the contracted kidneys has generally been smooth (Fig. 3, *A*), although in one dog gross lobulations were present (Fig. 3, *B*). Microscopically, changes not unlike those seen in human Bright's disease have been noted. The major abnormalities are shown in Fig. 4 and are contrasted with the normal appearance of the intact kidney from the same animal. The changes include: dilatation and atrophy of tubules; formation of casts within the tubular lumina; interstitial fibrosis; decrease in size of glomerular tufts; dilatation of Bowman's space with deposition of proteinaceous material therein; and thickening of glomerular

capsular epithelium and basement membranes. The intact kidneys have shown no consistent structural abnormalities. The majority of arterioles in both kidneys remained normal.

Clinically, the animals retained their general well-being and vigor. They demonstrated no evidence of edema-formation on an unrestricted diet; and neither hypertension nor progressive anemia developed. Only minimal elevations of plasma urea and creatinine levels occurred and these were commensurate with the over-all decrease in the combined values of glomerular filtration rates for both kidneys.

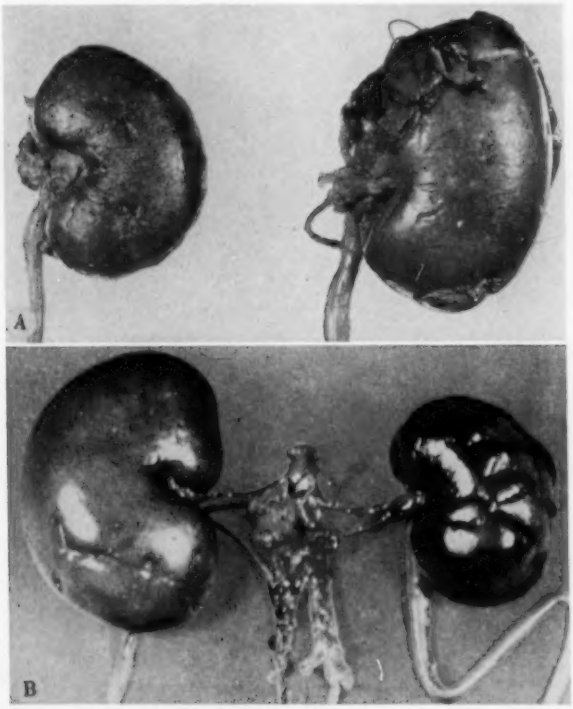

Fig. 3.—*A*, Contrasting appearance of the contracted kidney with the contralateral intact organ in a dog autopsied 9 months after induction of the lesion. The difference in weights was fourfold. The mass of the intact kidney is approximately 60 per cent of what it was in vivo. This is due to the fact that a heminephrectomy was performed two days prior to the death of the animal. *B*, Contrasting appearance of the contracted kidney with the intact kidney in an animal autopsied 5 months after induction of the lesion. The gross lobulations present in the contracted kidney were not noted in any of the other animals.

DISCUSSION

The technique described appears to offer a reproducible method of inducing chronic renal disease in dogs. The lesion is characterized by contraction of the kidney, the appearance of histologic abnormalities in persisting nephrons and a decrease in clearance values. Within the limits of the analogy between this

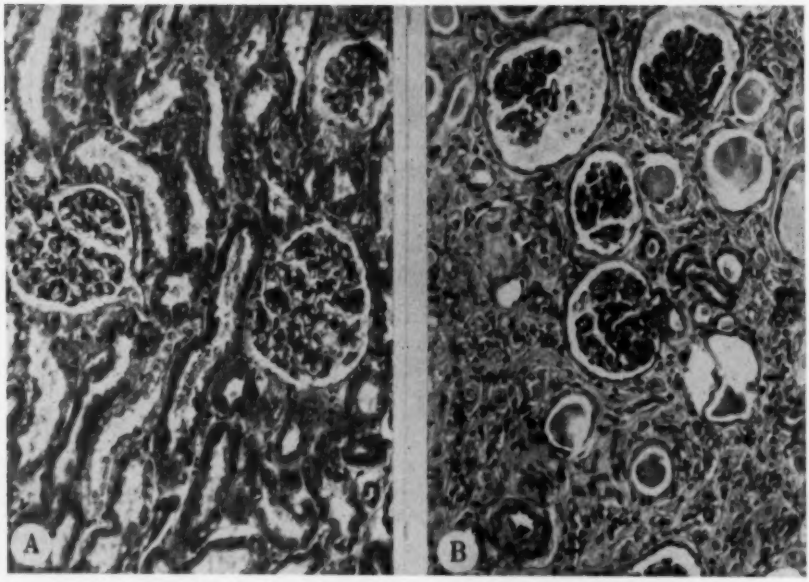

Fig. 4.—*A*, Control kidney from a dog whose opposite kidney was perfused with the aminonucleoside solution 5 months previously. This is a representative field showing normal glomeruli and tubules. Slight interstitial infiltration with chronic inflammatory cells can be seen in the otherwise normal kidney. *B*, The experimental (perfused) kidney from the same dog. The field is through a depressed area of the cortex. The tubules show cast formation and atrophy with interstitial fibrosis. The glomeruli are also affected, as evidenced by dilatation of Bowman's space and thickening of the capsule. (Magnification ×150; reduced ¼.)

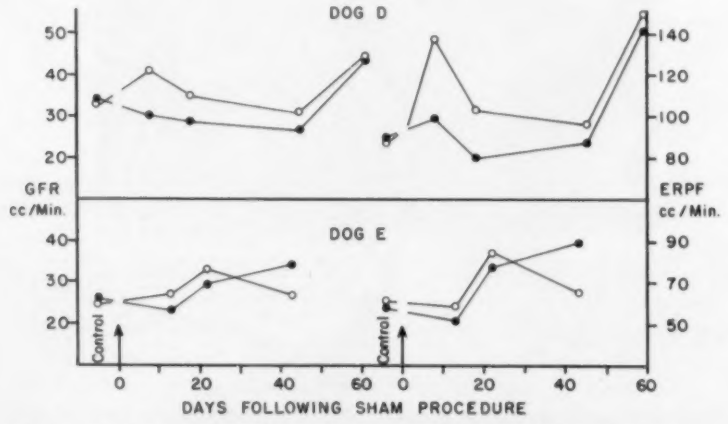

Fig. 5.—Serial bilateral renal function studies following unilateral perfusion of the anoxic kidney with a saline solution. Open circles = intact kidney; closed circles = experimental kidney.

lesion and that of chronic Bright's disease in man, the experimental model may aid in clarifying certain aspects of the functional capacity of the diseased human kidney.

The mechanisms by which the perfusion procedure lead to the development of a contracted kidney are unknown. Necropsy findings have shown no evidence of occlusion of either the renal artery or renal vein; and, in one dog with a severe lesion (glomerular filtration rate = 4 ml./min. experimental kidney; 38 ml./min. intact kidney), a translumbar aortogram performed within 2 weeks of the perfusion procedure demonstrated both renal arteries to be patent and to have approximately the same luminal caliber. The duration of renal anoxia appears to be one important contributory factor. Thus, it has been found that perfusion of the aminonucleoside solution through kidneys rendered anoxic for under 30 minutes is attended by the development of a less severe functional

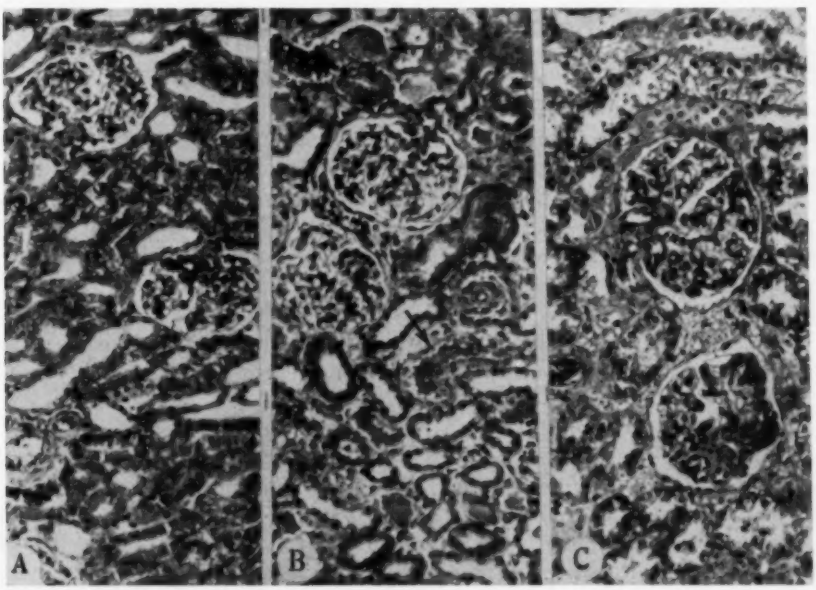

Fig. 6.—Serial histologic changes in a kidney subjected to a sham procedure. *A*, Biopsy of the experimental kidney in the control state showing normal glomeruli and tubules. *B*, Biopsy of the same kidney taken 14 days after perfusion with saline. Glomeruli are normal but some of the tubules contain casts and have vacuolated epithelium (see arrows). *C*, Section of the same kidney, 41 days after the "sham" injection, showing essentially normal glomeruli and tubules. (Magnification ×150; reduced ¼.)

and anatomic lesion. Similarly, when the collateral circulation to the kidney persisted during the perfusion procedure (as evidenced by bloody venous return through the renal vein catheter throughout the period of perfusion), the decrease in function was only moderate and unsustained. Anoxia per se, however, cannot be incriminated as the sole etiologic factor, for, in both dog and man, kidneys deprived of their blood supply for 90 minutes have been found to regain essential functional integrity.[5, 6] Moreover, preliminary observations

suggest that the combination of anoxia plus the perfusion procedure (using a saline solution without the aminonucleoside) will not induce the above-described lesion. In dogs subjected to this type of sham procedure (Fig. 5) an initial decrease in clearance values has been noted in the experimental kidney. However, over a 6- to 8-week period, renal function has typically increased to approximate that of the contralateral organ. Serial histologic studies on an animal subjected to a sham procedure are shown in Fig. 6. In the control state renal function was equal bilaterally, and the biopsy of the experimental kidney (Fig. 6, *A*) showed no histologic abnormalities. The second biopsy was obtained on the fourteenth day when the experimental kidney demonstrated functional impairment (creatinine clearance = 53 per cent of intact kidney). Swelling and degeneration of tubular epithelial cells were present (Fig. 6, *B*). The final biopsy was obtained on the forty-first day at which time functional recovery had occurred (creatinine clearance = 106 per cent of intact kidney). Only slight evidence of persisting morphologic abnormalities persisted (Fig. 6, *C*). At autopsy both kidneys were of normal size, and the experimental kidney demonstrated none of the changes seen in the animals perfused with the aminonucleoside solution.

The hypothesis is currently being investigated that the aminonucleoside, in the presence of sustained anoxia, is responsible for the development of the renal lesion. Some precedent for assigning a causal role to the aminonucleoside is found in the recent studies of Frenk and associates[4] and Fiegelson and coworkers.[5] Both groups have noted that the chronic administration of the aminonucleoside will induce a nephrotic syndrome in rats. Moreover, aminonucleoside nephrosis in rats is frequently associated with the development of progressive azotemia. What property or properties of the aminonucleoside may result in permanent alteration of nephrons, however, is unknown.

SUMMARY

A method has been developed for inducing unilateral chronic renal disease in dogs. The technique consists of perfusing the anoxic kidney with 100 ml. of isotonic saline containing from 0.75 to 3.0 Gm. of 6-dimethylaminopurine-3-amino-D-ribose. The outflow from the kidney during the perfusion is directed to the exterior through an inlying renal venous catheter, thereby preventing recirculation of the perfusate through the vascular bed of the contralateral kidney. The results of experiments to date indicate that this procedure will result in permanent impairment of function, marked histologic abnormalities, and ultimate contraction of the involved kidney. The contralateral (intact) kidneys have shown neither functional nor anatomic abnormalities.

The presence of one intact kidney and one diseased kidney in the same animal has provided an experimental model appropriate for investigation of the functional capacity of the contracted kidney.

We wish to thank Mr. Frank Deitz, Miss Claire Klein, and Miss Tove Kirkensgaard for invaluable technical assistance.

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COMPLEMENT FIXATION REACTIONS WITH DNA AND LEUKOCYTE MATERIAL IN SYSTEMIC LUPUS ERYTHEMATOSUS

Correlation With the L.E. Cell Phenomenon and the Clinical Status

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With the Technical Assistance of Mary H. Baker and Thelma A. Bradshaw

ALMOST simultaneously 4 reports have appeared within the past several months[1-5] which describe unique serologic reactions that can be developed in the serum of some patients with systemic lupus erythematosus (S.L.E.). These observations follow by almost 10 years the first identification by Hargraves and associates[6] of the L.E. cell phenomenon which very likely is the result of similar serologic abnormalities in this disease. The L.E. cell phenomenon has been repeatedly confirmed as a nuclear alteration pathognomonic of S.L.E.; its tissue counterpart, the hematoxylin staining body, has likewise been identified as a peculiar modulation in fixed tissue cell nuclei.

The recent serologic studies have disclosed that, in S.L.E., complement fixation reactions develop when cell nuclei, nucleoprotein, or desoxyribonucleic acid (DNA) have been used as the "antigen" in the reaction. The reactant factor or factors in the serum of these patients appear to be gamma globulin fractions which may be similar to or identical with the serum factor responsible for the L.E. cell phenomenon.

Since the latter is quite specific for S.L.E. it would be of interest to know whether the DNA and cell nuclei complement fixation tests are likewise so selective. The initial reports on these tests gave only a partial answer to this question. The controls selected invariably showed negative results but they were composed largely of "normal sera" or were obtained from "miscellaneous diseases showing high levels of gamma globulin." Two series contained sera from a number of patients with rheumatoid arthritis but almost no determinations have been made on other of the "collagen" diseases.

The purposes of this report will be to expand on the following: (a) to re-emphasize the fact that complement fixation reactions to DNA and leukocyte material (presumably primarily nucleoprotein) do develop in certain patients with S.L.E. and to correlate the results of the complement fixation tests with the L.E. cell phenomenon and the clinical status of the patient; (b) to test

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serologically a number of patients from most of the other accepted categories of connective tissue disease in order to further determine the specificity of these tests; and (c) to describe in some detail the sequence of clinical and serologic events that transpired in one patient with evolving S.L.E.

MATERIALS AND METHODS

DNA Isolation.—DNA was obtained from surgically resected normal human spleen. Nucleoprotein was separated by repeated homogenization of the tissue in a cold normal saline and Versene solution at pH 7.0. It was then dissociated by 30 per cent (w/v) sodium xylene sulfonate. After threefold dilution with distilled water, precipitated protein and other insoluble debris was removed by filtration with the aid of graded celites on sintered glass funnels. DNA was collected after repeated reprecipitation with 0.54 volume of isopropyl alcohol in the presence of 0.3 M sodium acetate at pH 7.0. Samples of DNA obtained by these methods were free of measurable protein or ribonucleic acid contaminants since acid hydrolysates showed no amino acids or uracil on paper-partition chromatography within the limits of detection. A 0.01 per cent solution of DNA in Kolmer saline was used in the test procedures.

Leukocyte Preparation.—The leukocyte material was obtained and prepared in the following manner. Twenty-five milliliters of blood was drawn from a patient with myeloid metaplasia, and collected in 0.5 ml. of a 5 per cent solution of Versene. The leukocytes were separated by the addition of commercial dextran, 9.0 ml. of a 6 per cent solution, packed by centrifugation, and resuspended to volume in normal saline. White cell counts revealed that approximately 0.176×10^{10} total leukocytes were present.* The cell suspension was then diluted with approximately 7 volumes of distilled water and, upon continued shaking, tenacious stringy material, resembling strands of DNA, appeared in the solution. The entire content was then centrifuged for 15 minutes at 1,300 g, the supernatant discarded, and the sediment was resuspended to the original volume (25 ml.) in Kolmer saline. It was stored in the refrigerator, stirred thoroughly before each test, and used without further dilution as the leukocyte "antigen" in the complement fixation procedures described below.

Complement Fixation Technique.—All reagents were diluted in Kolmer saline and each test was run as follows: to 0.1 ml. of the test serum (inactivated for 30 minutes at 56° C.) was added 0.15 ml. of Kolmer saline, 0.25 ml. of antigen (DNA 0.01 per cent or leukocyte material), and 0.5 ml. of guinea pig complement (2 full units). The suspension was incubated at 4° C. for 18 hours and subsequently warmed to 37° C. for 10 minutes. Then 0.5 ml. of a 2 per cent suspension of thrice washed sensitized sheep cells was added and the test was read after further incubation at 37° C. for 1 hour. A serum control was run with each test. The results were recorded as 4+, complete fixation of complement, through 0, no fixation (complete hemolysis). In all instances except one,† the tests when positive were 3+ to 4+ in degree. Antigen dilution, tenfold or a hundred fold, when tested in a few cases usually did not cause a significant decrease in complement fixation and in fact appeared to enhance it in some tests. Neither the DNA nor leukocyte material was anticomplementary in the concentrations used. One serum was anticomplementary on all occasions when it was tested.

RESULTS

Analysis of Clinical and Serologic Features in S.L.E.—As demonstrated in Table I, 7 of 20 patients with previous or current evidence of S.L.E. have shown positive complement fixation reactions to DNA and 5 of 16 to leukocyte lysate substance. All of the persons with positive tests had severe systemic

*Equivalent to roughly 12.0 mg. of DNA.
†Patient Ca.

clinical disease at the time of testing or had been through stormy periods within recent months. Two of the 7 died of their disease within a matter of days or weeks. On the other hand, among the 13 with negative complement fixation reactions 3 had severe clinical disease, although in 2 the disorder was largely confined to the kidneys with progressive uremia in each instance as the major clinical component.

TABLE I. COMPARISON OF CLINICAL SITUATION AT TIME OF STUDY WITH STRENGTH L.E. CELL PHENOMENON AND COMPLEMENT FIXATION REACTIONS

PATIENT	SEX	CLINICAL STATUS	L.E. CELL FORMATION	COMPLEMENT FIXATION	
				DNA	LEUKOCYTE
Do.	M	Severe	Strong	+	+
Su.	F	Severe to death	Strong	+	+
Fa.	M	Severe to death	Strong	+	+
Ca.	F	Severe	Strong	+	--
De.	F	Moderate	Weak	+	+
Og.	F	Moderate	Strong	+	--
Ga.	F	Severe	Strong	+	+
Wa.	F	Severe to death*	Weak	0	0
Si.	F	Severe	Strong	0	0
Be.	M	Moderate	Strong	0	0
Pe.	F	Moderate	Weak	0	--
Ho.	M	Moderate	Weak	0	0
Gi.	M	Moderate	Weak	ac†	ac†
Ev.	F	Moderate	Weak	0	0
An.	F	Moderate*	Negative	0	--
Se.	F	Severe to death*	Negative	0	0
Po.	F	Moderate	Negative	0	0
We.	F	Mild	Negative	0	0
Ro.	F	Remission	Negative	0	0
Rom.	M	Remission	Negative	0	0

*Chiefly renal involvement.
†Anticomplementary.

Analysis of this series of cases reveals a variable degree of correlation between the L.E. cell test and the complement fixation reactions to both DNA and leukocyte material. In all cases where the complement fixation tests were positive, L.E. cells were found repeatedly, even in sera that had been frozen for several months prior to study. In 7 other cases, however, L.E. cells were present, oftentimes repeatedly and in significant numbers, while the complement fixation tests remained consistently negative. Hence the L.E. cell phenomenon appears to be a more sensitive index of the presence of S.L.E. whereas the serologic reactions against DNA and leukocyte material suggest the presence of a severe disease state. The sole instance in which the L.E. cell test was negative and a serologic test positive will be described later.

Among the 6 cases labeled as S.L.E. in this series with negative L.E. cell formation at the time of this study, 2 were in clinical remission but had previously demonstrated L.E. cells and 1 was in relapse but had shown L.E. cells on several occasions in the previous year. Another had, primarily, renal localization of the L.E. disorder as proved by renal biopsy and subsequently at autopsy; the 2 remaining cases had many features of S.L.E. including variable leukopenia (1 case), chronic or variable biologic false positive serologic tests for syphilis (both cases), arthritis and arthralgias (both cases), vasculitis

by biopsy of muscle (1 case), and dermal manifestations (both cases). In all 6 of these persons with current absence of the L.E. cell phenomenon, the complement fixing responses were likewise negative.

The Effect of Serum Absorption Upon the L.E. Phenomenon.—Ten of the sera that showed the ability to induce L.E. cell formation were absorbed with DNA or with the human leukocyte material. Postabsorption study of the sera for its ability to form L.E. cells disclosed that in no instance was the phenomenon inhibited by the leukocyte material and in only one case (Do.) did DNA abolish or significantly diminish this test. The latter was confirmed by study of 6 sera obtained at different times in this patient. It is of interest to note in this respect that serial serologic studies in this patient disclosed a close temporal relation between the appearance of a positive L.E. cell test and a DNA complement fixation reaction (Fig. 1).

An impression was formed that on several occasions absorption by both the nuclear material and especially the DNA caused a slight quantitative reduction in the number of L.E. cells formed. Furthermore, many of the L.E. bodies formed after absorption in some cases seemed to retain coarse chromatin clumps which suggested that there was some qualitative inhibition of complete metamorphosis of the L.E. cell phenomenon.

Results of Complement Fixation Tests in Other Diseases.—As demonstrated in Table II, the serum from 53 persons with various diseases was tested for complement fixation reactions with DNA and most of them were also tested against leukocyte material. The reactions in all instances were negative. In the selection of these sera particular emphasis was placed upon securing samples from persons with other connective tissue and rheumatic diseases since it is in this group that S.L.E. will on occasion make its appearance. It is particularly significant that all the tests were negative in this select group.

TABLE II. TABULATION OF OTHER DISORDERS
(Negative Complement Fixation Reactions in All Instances)

DISORDER	NUMBER STUDIED
Rheumatoid arthritis	17
Rheumatoid spondylitis	2
Dermatomyositis and polymyositis	8
Scleroderma	4
Discoid L.E.	2
Polyarteritis	2
Arthritis with psoriasis	1
Gout	2
Miscellaneous (nonrheumatic) diseases*	15
Total	53

*Including 4 cases with chronic biologic false positive reactions, 2 with a true positive Wassermann reaction, and 3 with significant hyperglobulinemia.

Serial Observations in a Patient With S.L.E.—Do. is a 16-year-old boy whose initial symptoms appeared in September, 1956. In the ensuing 19 months he has demonstrated at one time or another many of the classical signs of S.L.E. Sporadic clinical features in his case have included a typical skin eruption, pericarditis, and probable myocarditis, leukopenia (950 white blood cells per c.mm. on one occasion), mild renal disease, pleurisy, fever, and buccal mucosal ulcerations. There was a history of "acute nephritis" in early childhood.

In Fig. 1 are recorded pertinent data concerning his clinical course and prednisone dosage as well as multiple studies of L.E. cell formation and complement fixation reactions over a 15-month period. Of particular interest in these serial laboratory studies are the following: (a) repeatedly negative L.E. cell tests during the early stages of the disease; (b) the emergence of a positive complement fixation test for the leukocyte lysate material on two occasions in May, 1957, when the tests for L.E. cells and DNA reactants were still negative; (c) simultaneous appearance in early June, 1957, of factors in the serum that induced L.E. cell formation and fixed complement in the presence of DNA. The reactions developed despite significant dosage levels of prednisone; (d) persistence of strongly positive reactions on all 3 tests throughout the next 7 months; (e) the appearance, in two separate fresh uncontaminated sera in February and March, 1958, of a strong anticomplementary response against sheep red blood cells with, however, persistence of a positive L.E. cell phenomenon; and (f) reversion to negative of both complement fixation reactions and the L.E. cell test 2 weeks later, apparently coinciding with significant clinical improvement on a higher steroid dosage.

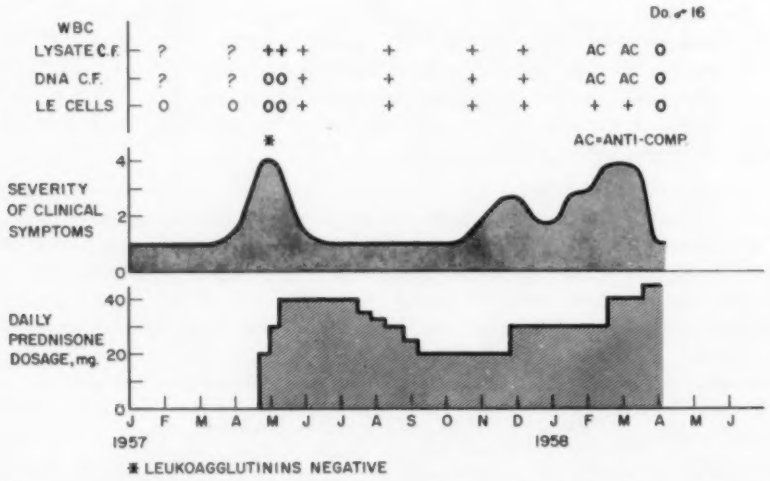
Do. of 16
WBC
LYSATE C.F. ? ? ++ + + + + AC AC 0
DNA C.F. ? ? 00 + + + + AC AC 0
LE CELLS 0 0 00 + + + + + + 0
*
AC=ANTI-COMP.
SEVERITY OF CLINICAL SYMPTOMS
4
2
0
DAILY PREDNISONE DOSAGE, mg.
40
20
0
J F M A M J J A S O N D J F M A M J
1957
1958
* LEUKOAGGLUTININS NEGATIVE

Fig. 1.—Comparison of serial serologic and L.E. cell tests, clinical status, and steroid dosage in one patient with evolving S.L.E.

DISCUSSION

There is accumulating an impressive body of evidence that one of the primary alterations in the disease S.L.E. is a morphologic and perhaps cytochemical derangement in the nuclear components of cells in several tissues and organs in persons afflicted with this disease. More than 25 years ago, Gross[7] recognized the hematoxylin stained bodies in tissue sections from cases of S.L.E. to be the result of morphologic alterations in nuclei. Considerable impetus was afforded for this concept by the discovery in 1948 of another nuclear modification, the L.E. cell phenomenon.[6] The L.E. cell test provides an in vitro counterpart of the tissue hematoxylin body and persists as a hallmark in the diagnosis of the disorder. Bits of evidence began to appear in the ensuing years that immunologic factors were involved in the induction of the observed

morphologic phenomena. These data included (1) the discovery of agglutinins for leukocytes in a high percentage of the sera from patients with this disease and (2) the localization in the gamma globulin compartment of the serum proteins of the factor responsible for the L.E. cell phenomenon. The most direct evidence for the presence of reactants against nuclear components in the serum of patients with S.L.E. has appeared, however, in the past few months with the demonstration by several immunologic techniques (complement fixation, hemagglutination, precipitation, and fluorescent antibody methods) of reactions between intact tissue nuclei, isolated cell nuclei, nucleoprotein, purified DNA or nuclear histone, and components in the serum of many patients with S.L.E.

Questions have been raised over the specificity of the L.E. cell phenomenon since morphologically identical bodies have been found in persons with otherwise typical rheumatoid arthritis and various types of drug sensitivity including penicillin allergy and hydralazine ingestion. There is no way at the moment to decide whether such cases are examples of a different disease or whether in fact they represent subclinical lupus which is brought to the level of clinical or laboratory recognition by one means or another. Further evidence such as that embodied in these recent serologic tests can, it is hoped, do much to resolve these problems.

Skepticism as to the specificity of these new complement fixation tests will undoubtedly develop. In an attempt to evaluate this question of specificity a rough analysis has been made of the total number of cases of S.L.E. and controls tested for reactants against DNA as obtained from the 4 recent reports[1-4] and the present series. These data are listed in Table III. They demonstrate that about one-half of the persons with S.L.E. will contain factors in their serum (?antibodies) that will react with DNA (or nuclear material) by the usual serologic tests. On the other hand, among the more than 320 control sera tested, including various conditions that manifest high globulin levels and representatives of almost all of the accepted divisions of "collagen" disease, the tests have been uniformly negative. It can be concluded that the specificity of the DNA complement fixation test rests upon firm ground.

TABLE III. TOTAL CASES TESTED FOR DNA REACTANTS
Summary from Recent Literature (See Text)

	NUMBER OF CASES	NUMBER POSITIVE	PER CENT POSITIVE RESULTS
Systemic lupus erythematosus	73	35	48
Other "collagen" diseases (largely rheumatoid arthritis)	89	0	0
Other diseases	108	0	0
"Normal" controls	125+	0	0

From information abstracted from the report by Seligman and Milgrom[4] plus that from our cases, it can be predicted that about 70 per cent (13 of 18) of persons with acute or severe S.L.E. will show a positive result.

Is the serum factor which is responsible for the L.E. phenomenon identical with that which induces the complement-fixing reactions, or are there multiple factors involved? A final answer to this question cannot be given at the present time but the current evidence seems to favor the latter. The cross-absorption experiments conducted by Robbins and associates[1] disclosed that absorption of active sera with whole isolated nuclei significantly reduced their ability to form L.E. cells, but failed to alter fixation with DNA. In contrast absorption with DNA significantly reduced the ability of the sera to fix complement with DNA without altering their ability to react with nuclei or to form L.E. cells. We have not conducted cross-absorption experiments but have studied the effect of serum absorption by either DNA or leukocyte lysate material on the L.E. cell phenomenon. Our leukocyte material probably was composed chiefly of nucleoprotein. It was prepared by a different technique from that of Robbins and co-workers and it did not inhibit the subsequent induction of L.E. cells by absorbed sera, but did appear to alter the morphology of the subsequent L.E. body.* On the other hand, the L.E. phenomenon was almost completely inhibited in one of our 10 active sera after absorption with DNA. These results suggest that separate substances are present in L.E. serum which may react against nuclei or several components thereof. Further proof for this hypothesis is afforded by an analysis of the serial studies reported on the patient depicted in Fig. 1. In this case it appears that at least 3 reactant factors can be dissected apart in time. It is likely that the situation may be even more complex than stated above so that other nuclear "antigens" will likely be described in the future. A suggestion in this direction is found in the notation by Robbins and associates[1] that 2 of their strongly positive sera demonstrated a low titer complement fixation response against nuclear histone.

The reported fact that L.E. serum will react against cell nuclei or DNA obtained from various organs and from species as far removed as the salmon likely attests more to the biologic uniformity of these substances than to the lack of specificity of the serum reactant factor in the L.E. patient.

It has been our impression that in addition to a minor quantitative decrease in the number of L.E. cells developed in some cases after absorption of active sera with DNA or leukocyte material there is also a qualitative morphologic difference in the formed L.E. body. It would be most interesting to investigate this feature with the rather precise cytochemical methods recently reported by Godman and Deitch.[8]

It would be difficult to predict what these recent serologic observations will contribute to the ultimate unfolding of the multisystem disease, lupus erythematosus. It may be that the factors involved are only end result reaction products of a more basic defect yet to be clarified. It is safe to conclude, however, that their discovery constitutes a significant advance in the attempt to unravel the intricacies of S.L.E. Furthermore, they appear to focus more and more attention on the nucleus as a prime mover in this disease.

*The fact that such a preparation can dissociate one reaction from the other, i.e., L.E. phenomenon from complement fixation, is of considerable significance although it must be admitted that the difference may be a quantitative rather than a qualitative one.

SUMMARY

Complement-fixing reactants against purified desoxyribonucleic acid or a lysate of human leukocytes have been found in the sera of 7 out of 20 patients with systemic lupus erythematosus. All of these patients had positive L.E. cell tests as did several others in which the serologic tests were negative. In general these tests indicated a severe form of disease and in one case with evolving S.L.E. it was possible to dissociate the 3 reactions over a 16-month period. The complement fixation tests were negative in all of over 300 cases other than S.L.E. abstracted from the recent literature and from this series. Included in this group was a strong representation from almost all of the other accepted catagories of connective tissue disease.

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EFFECT OF INTRAVENOUS FIBRINOLYTIC ENZYMES ON THE VEGETATIONS OF EXPERIMENTAL BACTERIAL ENDOCARDITIS

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BACTERIAL endocarditis is one of the dreaded diseases of man. Although antibiotic drugs have increased the recovery rate from this infection, the mortality and morbidity rates have remained significant. For this reason there has been a continuing search for means of rendering antibiotic treatment more effective in this disease.

The organisms which commonly cause bacterial endocarditis are readily attacked by chemotherapeutic drugs. Nevertheless, the bactericidal actions of these agents in the blood may be hindered by the fibrin barrier of the endocardial vegetations even though the blood concentration of the drug appears to be adequate. This fact suggests that measures which would reduce the fibrinous masses of the lesions should be of adjunctive value in the chemotherapy of the infected endocardium. Furthermore, the high fibrin content of the valvular excrescences renders them particularly susceptible to activating agents of the proteolytic plasminogen-plasmin enzyme system. This system is effective against fibrin. Streptokinase activates this reaction.[1]

In order to observe the effects of streptokinase on the course of experimental bacterial endocarditis in dogs, the following experiments were carried out.

METHODS

Mongrel dogs (7 to 18 kilograms) were anesthetized with sodium pentobarbital. The right carotid artery was exposed and an infant-sized laryngeal biopsy forceps (Storz N-8490) was inserted into the vessel and advanced to the level of the aortic valve. Tissue was then removed from the area. With this method, the coronary cusps of the aortic valve or the anterior leaflet of the mitral and chordae tendineae of the mitral valve were incised or torn. This aortic or mitral valvular injury occurred with approximately equal frequency. Twenty-five per cent of the animals died within 7 days of the operation, either from congestive heart failure resulting from valvular damage or from hemorrhage and shock due to accidental intimal damage to the aorta.

It was assumed that valvular injury had occurred when loud systolic or diastolic murmurs became audible. The operation was considered unsuccessful in the absence of

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a murmur. Procaine penicillin (400,000 units) and streptomycin (0.5 Gm.) were administered intramuscularly after the procedure. The animals received no more antibiotic treatment during the study.

Control observations were conducted for a week. The pulse rates, rectal temperatures, and quality of the murmurs were recorded daily. Each dog was weighed regularly. Blood cultures were obtained, and random animals were followed with erythrocyte sedimentation rates and hematocrit determinations. The animals that developed inanition, temperatures of 39.5° C. or above, or signs suggesting heart failure were eliminated from the study.

The dogs that withstood the valvular damage with no evidence of complicating infection were then subjected to streptococcal infection. We prepared bacterial suspensions of 4-hour subcultures (of 16-hour cultures) of *Streptococcus mitis* (JH-26, Lancefield D) in dextrose-beef heart infusion broth. The animals were given 1 to 4 intravenous injections of the bacterial suspension on succeeding days. The development of persistent fever of 39.5° C. or more in the presence of a conspicuous murmur was considered indicative of active bacterial endocarditis. The infection was always accompanied by a positive blood culture. Anorexia, lassitude, and weight loss were usually present. A change in the intensity and quality of the murmurs sometimes occurred during the febrile periods.

The animals that developed bacterial endocarditis were divided into two general groups: (1) control animals and (2) animals treated with fibrinolytic agents. These two groups were then subdivided into the dogs that succumbed to the infection, and those animals that were dispatched at specific times following the appearance of endocarditis.

The fibrinolytic enzymes were a preparation of streptokinase (155,000 units) and streptodornase (66,000 units).* The substance was administered in saline intravenously at approximately 12-hour intervals for 6 injections. In some instances additional injections were given. The average amount in each infusion was 465,000 units of streptokinase and 198,000 units of streptodornase. Fibrinolytic activity of the dogs' sera was determined by clot lysis on incubation (for 1 hour at 37° C.) of blood obtained 15 minutes after enzyme administration. Blood fibrinolytic activity before enzyme treatment was also noted.

All of the hearts were examined after death. The chambers were opened after fixation in 10 per cent formalin to avoid dislodging endocardial vegetations. Special attention was given to the size and site of the valvular lesions. Sections for microscopic study were prepared from all hearts whether or not gross lesions were apparent. The lungs, liver, spleen, and kidneys were examined in some instances.

RESULTS

Forty-nine dogs with significant heart murmurs comprised the total series of experimental animals. Of these animals, 7 were followed without bacterial injections to observe the natural course resulting from valvotomy. Three of these dogs exhibited aortic diastolic murmurs; they survived an average of 4 weeks and died in acute pulmonary edema. Four dogs (with systolic murmurs which lessened in intensity or disappeared during the period of observation) did not appear ill, and when dispatched 3 to 7 weeks after operation showed scant evidence of injury to the valve structures. These dogs were considered operative failures. Thereafter, care was taken to exclude the animals that failed to exhibit persistent murmurs from the enzyme-treated group.

Of the 42 animals subjected to valvular trauma and then inoculated with *Streptococcus mitis*, 27 exhibited evidence of bacterial endocarditis at necropsy. In general, animals with the most prominent murmurs were more likely to

*Streptokinase-streptodornase (Varidase) was kindly furnished by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

develop the lesions. Combined aortic and mitral valvular disease occurred more frequently than solitary valve involvement. The location of the infection, whether aortic, mitral, or both, seemed to have no predictable effect on the clinical course or on the response to intravenous fibrinolysis.

Effect of Streptokinase-Streptodornase on Survival Time.—Data relative to the survival of treated and untreated endocarditis are shown in Table I. In general, the dogs that received intravenous enzymes survived only 3 days longer than the untreated animals. This difference is not satistically significant due to the small numbers of subjects and the wide variation in survival times. However, it was not expected that longevity would be influenced by the treatment because antibiotics were not administered to eliminate the bacterial valve infections.

TABLE I. COMPARISON OF THE LENGTH OF SURVIVAL BETWEEN THE CONTROL AND STREPTOKINASE-TREATED ANIMALS

	NUMBER OF DOGS	AVERAGE LENGTH OF SURVIVAL AFTER FEVER	RANGE OF DURATION OF SURVIVAL
No treatment	12	9.3 days	1-16 days
Streptokinase-treated	9	12.6 days	2-23 days

Effect of Enzyme Treatment on the Size of Vegetations.—The size of the valvular vegetations in the animals that were dispatched, or that died soon after enzyme treatment, was compared to those in the control dogs at a comparable time after the onset of fever. In most of the *untreated* dogs there were large, florid vegetations on the mitral or aortic valves, measuring up to 9 by 20 mm., but some were small. The appearance of the valvular lesions in dogs which received streptokinase-streptodornase was often small, but the heart of one enzyme-treated dog showed an aortic vegetation 10 by 5 mm. together with a large mitral vegetation, and 2 other specimens exhibited rather large fibrinous masses on the mitral leaflets (cf. Table II). In 2 instances, there were areas of roughened valvular endocardium which grossly indicated intense endocardial inflammation, although vegetations were entirely absent, as shown in Figs. 1 and 2. Microscopically these areas were heavily colonized by streptococci.

TABLE II. COMPARISON OF THE SIZE OF THE VEGETATIONS BETWEEN THE CONTROL AND STREPTOKINASE-TREATED ANIMALS

	NUMBER OF DOGS	SIZE VEGETATIONS IN GREATEST DIAMETER		
		SMALL (LESS THAN 2 MM.)	MODERATE (3-6 MM.)	LARGE (MORE THAN 6 MM.)
No treatment	14	3	1	10
Streptokinase-treated	11	5	2	4

Only 4 of 11 enzyme-treated animals had "large" vegetations, whereas 10 of 14 untreated dogs had large lesions. However, statistical evaluation of these results using the Chi-square test failed to show a statistically significant difference between the incidences of large vegetations ($p > 0.05$). Therefore, we must conclude that with these small numbers of animals the differences

in vegetation sizes between the treated and untreated groups are dictated by chance. On the other hand, if the same differences in the lesions had proved to be present in 12 additional animals, the statistical results of the Chi-square test would become significant.

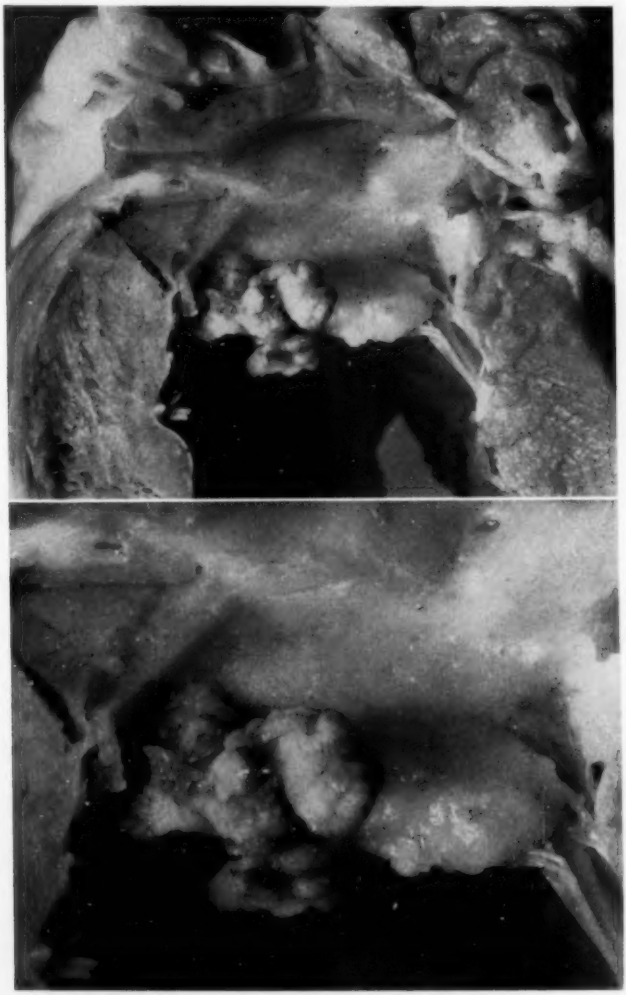

Fig. 1.—*A*, The mitral valve in the heart of a dog with *Str. mitis* endocarditis, showing the typical large, fungating vegetation in relation to adjacent tissues. *B*, Enlarged photograph of the same fibrin vegetation.

On histologic examination of the heart valves, bacteria were identified near the surfaces of the fibrinous deposits in both treated and untreated

groups, and areas of interstitial myocarditis were frequently noted in the ventricular muscle.

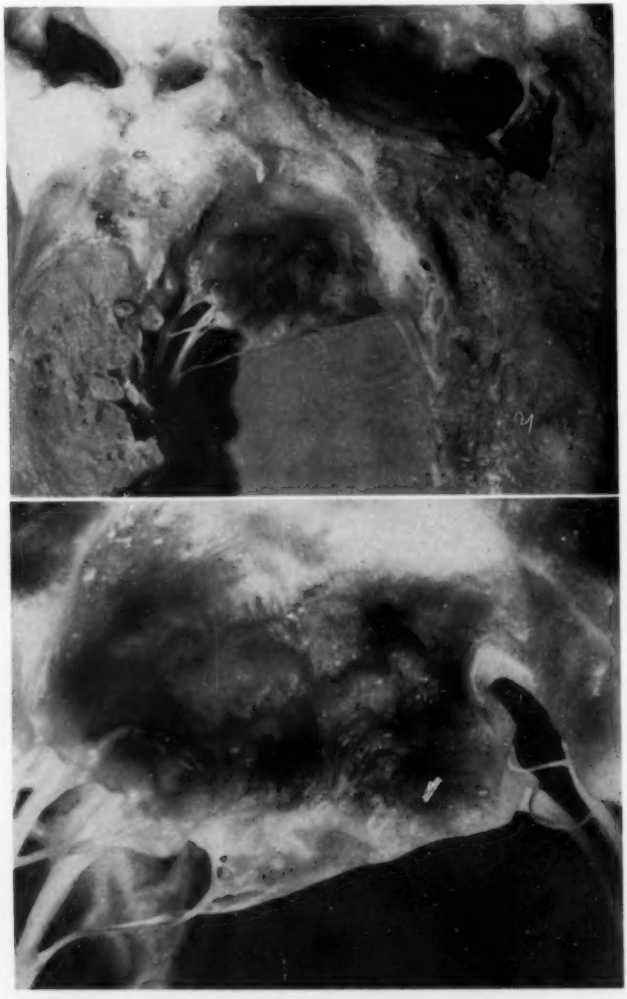

Fig. 2.—*A*, The mitral valve in the heart of a dog with *Str. mitis* endocarditis treated with streptokinase-streptodornase (*cf.* text). The valve has been damaged and infected, but there is no discernible fibrin deposition or vegetation. *B*, A closer view of this mitral valve.

Influence of the Enzymes on Fibrinolysis.—Six of the 13 dogs treated with streptokinase-streptodornase showed complete in vitro clot lysis in 1 hour, while 5 others demonstrated partial clot lysis. In 2 dogs, the tests were not performed. No consistent differences were discerned in the valvular lesions of these animals.

Hemorrhage did not occur as a complication of enzyme therapy. One dog bled into the upper mediastinum, although the accident may have resulted from the surgery. The hematocrits were constant during the period that proteolytic substances were administered. Two animals without endocarditis were given average doses of streptokinase-streptodornase. Aside from slight fever, the dogs had no untoward reactions from the drug, and post-mortem examinations were negative.

DISCUSSION

Attempts to produce bacterial endocarditis by Lillehei's method,[3] using bilateral femoral fistulas rather than aorta-vena caval shunts, were successful in only 2 of 40 dogs. The valvotomy proved more reliable inasmuch as 63 per cent of the animals receiving *Str. mitis* after valvotomy developed active endocarditis. In contrast, the facility with which the valvular infection was produced must be balanced against the uncertain nature of the resultant valvular lesion, the lack of identity of the injured valve, and the disappointing postoperative mortality of 25 per cent in the succeeding 7-day interval. It is interesting that several animals developed nonbacterial valvulitis of the tricuspid valve. Highman and associates[2] reported tricuspid valvulitis in many of their dogs where direct trauma to the valve had not occurred. The lesion may have been related to increased work load and cardiac stress.[5] It is not clear whether the mitral endocarditis in our animals resulted from infection of valves subjected to direct instrumental trauma, or to endocardial damage resulting from changing cardiac hemodynamics. Both factors may have been concerned.

Experimental studies have indicated that the structure of the vegetations of bacterial endocarditis affords protection to the infecting organisms. The vegetations are formed by a large central necrotic zone of acellular debris, adjacent to which are colonies of bacteria surrounded by a predominantly fibrinous covering.[6] In 1938, Friedman and co-workers[7] reported that fibrin was an ideal medium for the growth of *Streptococcus viridans*. They also showed that cultures of this organism in fibrin-coated capsules were not invaded by leukocytes; these cultures became sterile. More recently, Hunter[8, 9] has demonstrated that bacteria enclosed within blood clots were sealed from their environment to the extent that the organisms were protected from antibiotic substances in the surrounding blood.

Clinical data have generally supported these experimental studies. Viable bacteria have been found in valvular vegetations after optimum treatment and apparent cure,[12] and the mortality of the disease remained relatively high despite adequate chemotherapy.[10, 11] This fact suggests that measures capable of disrupting the protective fibrin layer of the vegetations should augment the effectiveness of chemotherapy and host resistance factors in controlling the infection. Further valvular damage should then become less frequent. For these reasons anticoagulant drugs have received an extensive trial in this disease. Heparin was used in conjunction with sulfonamides, but the combination proved clinically ineffective and even dangerous.[13, 14] Dicumarol was

similarly unsuccessful. In an autopsy study of patients receiving anticoagulants, Priest[14] found no difference in the amount of fibrin comprising valvular vegetations between the treated and untreated patient groups.

Mayer[15] has recently reported the use of hyaluronidase to render the fibrin barrier of infected heart valves more permeable to antibiotics. A favorable therapeutic effect was noted. However, the report included only 2 cases.

In contrast to the disappointing results with adjunctive anticoagulant treatment, the experimental evidence suggests that streptokinase (through its high fibrinolytic activity) may be of supplemental value in the antibiotic therapy of bacterial endocarditis. Local injections of the enzyme have been successful in liquefying fibrinous exudates. Other work has shown the ability of the enzymes to lyse arterial thrombi and to prevent postoperative adhesions.[1, 16] Streptokinase has been administered intravenously to man without serious side effects.[17, 18]

In the present report, intravenous streptokinase-streptodornase in some instances appears to have reduced the size of vegetations in experimental infective endocarditis in dogs. Although the inflammatory responses in the valves were not significantly altered in the control dogs as compared to those receiving enzymes, nevertheless, reduction of the vegetations suggests that fibrin deposition was impeded in the presence of active infection. Since there is difficulty in quantitating the size of the vegetations, and because the data are not statistically significant at the 5 percentile level, conclusions from this study can only be tentative. Antibiotics were not given, and, as expected, the fibrinolytic enzymes had no definite effect on the time course of the disease.

SUMMARY

1. Bacterial endocarditis developed in 27 of 42 dogs following valvular trauma and the intravenous injection of *Streptococcus mitis*.

2. The effects of intravenous streptokinase-streptodornase on longevity of the disease and on the size of bacterial vegetations were observed.

3. The proteolytic enzymes did not prolong life in the absence of antibiotic therapy.

4. There was suggestive evidence that the size of the valvular vegetations was reduced by intravenous streptokinase-streptodornase treatment.

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THE ERYTHROPOIETIC STIMULATORY ACTIVITY OF BATYL ALCOHOL

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THE mechanisms governing red cell production have been the subject of intensive investigation in many laboratories during the past few years. These studies[1] have confirmed the existence of humoral stimulatory factors which exert important physiologic and pathophysiologic regulatory control over erythropoiesis. Our observations indicate that there are two such agents which control, respectively, the rate of erythroblastic cellular division and the synthesis of hemoglobin.[2, 3] Although the chemical identities of the plasma erythropoietic factors are not known, current information[3] suggests that the factor concerned with cellular division is most likely a lipid and the other, which augments hemoglobin formation, is probably protein in nature.

In 1941, Holmes and his co-workers[4] isolated batyl alcohol from the non-saponifiable fraction of bovine yellow bone marrow. This compound, the monoglycerol ether of n-octadecyl alcohol [$CH_3(CH_2)_{17}$-O-CH_2-CH(OH)-CH_2(OH)], had previously been found only in marine organisms. Sandler, who also obtained batyl alcohol from yellow bone marrow, reported, in 1949, that the material stimulated erythropoiesis when administered by subcutaneous injection to normal and benzene-treated rats.[5] He also described reticulocyte responses in normal human subjects who were given an optically inactive synthetic preparation of batyl alcohol. The chemical and physical properties common to this compound and the thermostable, ether-soluble plasma erythropoietic factor[3] suggested a possible relationship between these two substances. Further studies on the hemopoietic effects of batyl alcohol were of interest, and it is the purpose of this communication to report the results of some observations with this material.

MATERIALS AND METHODS

Female Wistar strain rats weighing approximately 160 grams were placed in 3 groups of 6 animals each. One group was given 20 subcutaneous injections of 2 ml. of

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peanut oil containing 12.5 mg. of racemic batyl alcohol* over a period of 4 weeks (daily, Saturdays and Sundays excepted). In a second group the dose was doubled over the same period of time. The third group received peanut oil only and served as the controls.

Hematologic studies consisted of hemoglobins determined by the cyanmethemoglobin technique, microhematocrits, hemacytometer erythrocyte and leukocyte counts, platelets counted with the phase microscope, and reticulocytes per 1,000 erythrocytes enumerated on dried brilliant cresyl blue cover slip films counterstained with Wright's stain. All values were obtained prior to the injections and every 7 days thereafter with the exception of the reticulocytes which were counted twice weekly throughout the 6-week period of observation. Cell diameters were measured on Wright's stained cover slip films with a micrometer disk and Price-Jones curves were constructed. Erythrocyte osmotic fragilities were determined by the direct cell enumeration technique previously described using red cell pipettes and hypotonic salt solutions as diluents.[6] At the conclusion of the injection period 3 rats in each group were killed and femoral marrow was examined according to a method previously described.[7] The remaining animals were observed for an additional 2 weeks to evaluate the return to normal hemic equilibrium.

TABLE I. STANDARD DEVIATION IN THE ERYTHROCYTES (MILLIONS PER CUBIC MILLIMETER)

	BASE LINE		1 WEEK		2 WEEKS		3 WEEKS		4 WEEKS	
GROUPS	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Batyl alcohol (25 mg. per day)	6.84	0.53	7.79	0.72	8.77	0.54	8.81	0.79	9.02	0.41
Batyl alcohol (12.5 mg. per day)	6.88	0.47	6.82	0.39	7.52	0.35	7.82	0.48	8.52	0.71
Peanut oil	7.17	0.40	6.89	0.31	6.88	0.19	7.19	0.29	7.16	0.28

TABLE II. STANDARD DEVIATION IN THE RETICULOCYTES (PER CENT OF ERYTHROCYTES)

	BASE LINE		1 WEEK		2 WEEKS		3 WEEKS		4 WEEKS	
GROUPS	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Batyl alcohol (25 mg. per day)	3.2	0.5	10.4	1.0	12.1	0.5	12.4	1.3	11.6	0.9
Batyl alcohol (12.5 mg. per day)	2.8	0.5	8.5	0.3	9.8	0.9	10.9	0.5	10.2	0.7
Peanut oil	3.3	0.4	3.4	0.9	3.1	0.7	3.3	0.5	3.5	0.6

RESULTS

Erythropoietic stimulation was manifested in the animals injected with batyl alcohol by erythrocytosis and reticulocytosis (Fig. 1 and Tables I and II) plus myeloid erythrocytic hyperplasia (Fig. 2). There was no associated increase in the hemoglobin or hematocrit levels (Fig. 1), and the microcytes responsible for the erythrocytosis were evident on the stained films and demonstrated graphically by Price-Jones curves (Fig. 3). The erythrocytosis and reticulocytosis were more marked and appeared earlier in the animals receiving 25 mg. of batyl alcohol per day than in those injected with half this amount (Fig. 1). Red blood cell counts showed a progressive increase in

*Batyl alcohol was kindly supplied by Mr. F. O. S. Spencer of George A. Breon & Co., New York, N. Y. The batyl alcohol was extracted in ether from the aqueous suspension in which it was received, the ether evaporated, and the crystalline material dissolved in warm peanut oil to form a cloudy solution of 6.25 mg. batyl alcohol per milliliter of peanut oil.

both groups throughout the injection period while the reticulocyte levels reached plateaus by the end of the second week. At the termination of the injections, decreased erythrocyte osmotic resistance was evident in the rats

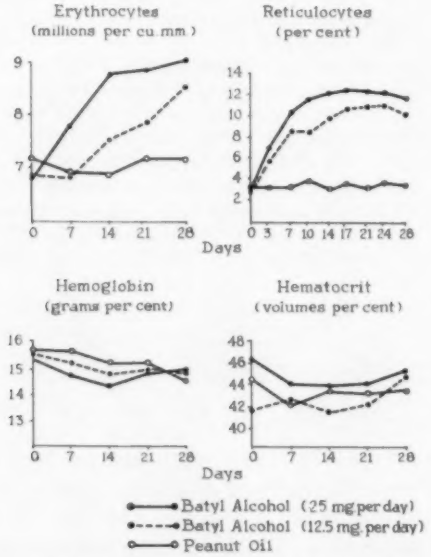

Fig. 1.—Erythrocytosis and reticulocytosis in rats injected with batyl alcohol without increases in the hemoglobin or hematocrit levels. Average determinations of 6 animals in each group.

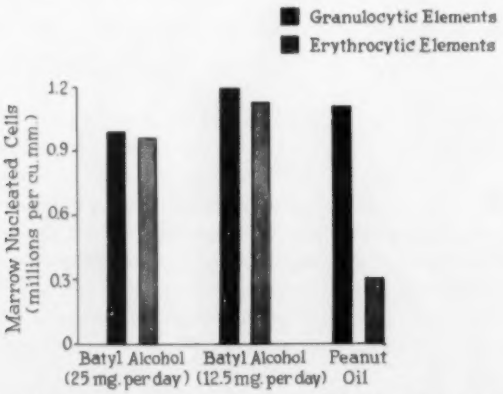

Fig. 2.—Myeloid erythrocytic hyperplasia in the recipients of batyl alcohol. There was a roughly proportional increase in all recognizable stages of nucleated erythrocyte development. Average counts of 3 rats in each group.

given the batyl alcohol (Fig. 3). Two weeks after the injections were discontinued, all determinations in the remaining animals had returned to normal base-line levels.

In addition to the evidence of accelerated erythropoietic activity induced by the administration of batyl alcohol, these animals also exhibited an increase in their platelet counts (Fig. 4 and Table III). Although not marked, the thrombocytosis was definite, manifested essentially the same relationship to the daily dose as did the erythrocytosis, and had returned to normal levels one week after the injections were stopped. Consistent and significant leukocytosis was not noted in these animals (Fig. 4) nor were the myeloid granulocytic elements increased in number (Fig. 2).

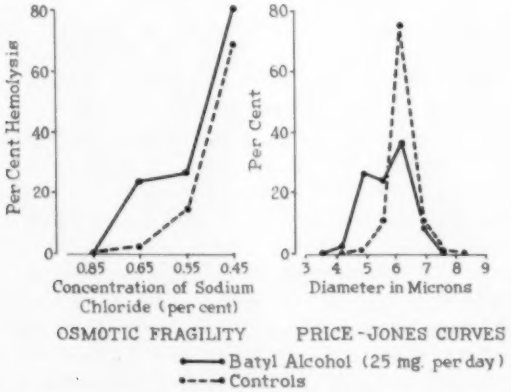

Fig. 3.—Microcytosis and increased osmotic fragility in recipients of batyl alcohol. Composite curves of osmotic fragility and red cell diameter distribution (Price-Jones) of 6 rats receiving 25 mg. of batyl alcohol per day and the same number of control animals at the end of the 4-week injection period.

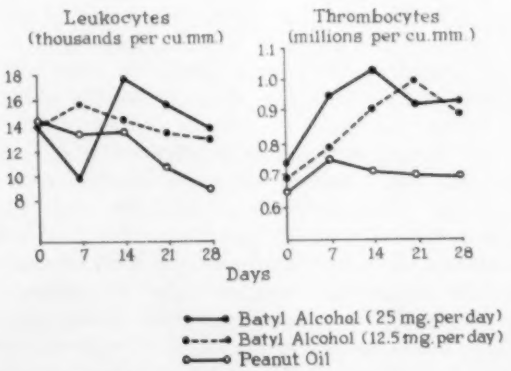

Fig. 4.—Thrombocytosis in recipients of batyl alcohol without consistent or significant increases in their leukocyte counts. Average determinations of 6 rats in each group.

The hematologic studies in the control group injected with peanut oil were constant throughout the entire experimental period. All animals remained healthy, gained weight normally, and demonstrated no local or general adverse effects from the injections.

TABLE III. STANDARD DEVIATION IN THE THROMBOCYTES (MILLIONS PER CUBIC MILLIMETER)

GROUPS	BASE LINE		1 WEEK		2 WEEKS		3 WEEKS		4 WEEKS	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Batyl alcohol (25 mg. per day)	0.738	0.150	0.957	0.167	1.030	0.022	0.910	0.077	0.929	0.093
Batyl alcohol (12.5 mg. per day)	0.691	0.116	0.790	0.206	0.913	0.185	1.000	0.087	0.894	0.082
Peanut oil	0.650	0.125	0.752	0.093	0.708	0.114	0.704	0.119	0.697	0.089

DISCUSSION

Our results are in agreement with those of Sandler[5] and confirm the erythropoietic stimulatory effect of batyl alcohol in normal rats. The much larger doses of batyl alcohol used in our experiments than those employed by Sandler presumably account for the greater increase in erythrocytes and reticulocytes than he observed. This apparent relationship between dosage and the degree of response in the recipients was suggested by Sandler's studies and is supported by our observations. As shown in Fig. 1, beginning erythrocytosis was not evident at the end of the first week of injections in the animals given 12.5 mg. of batyl alcohol per day, whereas the rats receiving twice this daily amount had an average increase in red cells of approximately 1,000,000 per c.mm. However, after 3 additional weeks the mean augmentations in erythrocytes per c.mm. over their respective base-line values were 1,640,000 in the former group and 2,180,000 in the latter. A similar disparity was also noted in the reticulocyte responses but of smaller magnitude. The type of response observed in the experiment described above may, however, be of greater significance in pointing toward such a relationship.

We have yet to study an active plasma, regardless of the experimental or clinical conditions under which it was obtained, that did not possess erythropoietic stimulatory activity attributable to both thermostable and thermolabile factors. Their combined effect on cellular division and hemoglobin synthesis of normal recipient animals results in the production of increased numbers of normal cells. Thus, the response to active whole plasmas or extracts which have been boiled for only short periods of time is characterized by an increase in all erythroid determinations. Active plasmas which have been processed by boiling for 30 minutes or the ether-soluble fractions of such plasmas contain only the factor concerned with erythroblastic cellular division and elicit in recipients a singular type of erythropoietic response characterized by the formation of microcytes without associated increases in the circulating hemoglobin or red cell mass,[2,3,6-9] an observation which has also been described by others.[10,11] The microcytes are readily discerned on stained blood films and easily demonstrable by Price-Jones curves. The restoration of normal erythrocytic equilibrium is prompt after the plasma extract injections are discontinued. Recent studies have shown that this phenomenon is apparently due to impaired viability of the microcytes produced in response to a particular stimulus as evidenced by their decreased resistance to lysis in hypotonic salt solutions.[6] Since this fragility abnormality is not

evident in situations involving increased activity of both plasma factors, it is probably the result of alterations in cell size and/or shape secondary to accelerated erythroblastic division in the absence of a stimulus for enhanced hemoglobin synthesis rather than due to a specific action of the thermostable factor.

The erythrocytic responses in normal rats to the administration of batyl alcohol were identical in all respects, demonstrable by the methods used, to those induced by the thermostable plasma erythropoietic factor.

Stimulation of erythropoiesis by anemic or by hypoxic hypoxia or by the administration of cobalt ion,[12] which probably acts by interfering with oxygen transport, has been shown to be mediated through humoral factors. The erythropoiesis stimulatory effect of batyl alcohol might be explained on the basis of: (1) Enhanced plasma erythropoietic activity, either through increased production of the humoral factors or antagonistic action on counteracting or inhibitor substances. (2) An action on erythropoiesis unrelated to that of the humoral factors which have been studied. (3) Identification of batyl alcohol with the thermostable factor or close relationship to it, possibly as precursor material.

The first hypothesis is susceptible of experimental proof which is not yet available. It seems unlikely because hypoxia, or interference with oxygen metabolism, which is the sine qua non for enhanced plasma erythropoietic activity in all other situations would not be expected to result from the administration of a compound such as batyl alcohol. Moreover, if batyl alcohol did in some way affect oxygenation in such a manner as to stimulate erythropoiesis, increases in the factors responsible for both enhanced hemoglobin synthesis and accelerated erythroblastic division would be expected to occur. The stimulus of hypoxia, in subjects capable of response, is always followed by elevation of both hemoglobin and erythrocyte values. Nevertheless, it is conceivable that batyl alcohol might inactivate an inhibitor of the thermostable factor, and more information is needed before this question can be answered.

The theory that batyl alcohol acts independently of the plasma erythropoietic factor is at present merely an explanation by exclusions. It should be subjected to test by assaying for increased activity the plasma of animals made polycythemic by means of batyl alcohol and by attempts to relate chemically the thermostable factor and batyl alcohol. If both of these experimental approaches should yield negative results, some other explanation for the action of batyl alcohol on erythropoiesis, not mediated through plasma humoral factors, should be sought.

Evidence currently at hand favors the third hypothesis, that of a close chemical relationship between batyl alcohol and the thermostable factor. Both stimulate division of erythrocyte precursors without augmenting hemoglobin production; both are resistant to heating to 100° C. for prolonged periods; they appear to be of comparable molecular size; batyl alcohol is a monoglycerol and the thermostable factor in plasma also appears to be a lipid.

In addition to its erythropoietic activity, batyl alcohol has other effects on hemopoiesis. There are a few reports[13-15] indicating that this alkoxyglycerol plays a role in leukopoiesis and possibly thrombopoiesis. We were unable to confirm the granulopoietic activity of batyl alcohol in the experiment described above, but the failure would appear to have been due to insufficient dosage. However, an increase was noted in the circulating thrombocytes in rats injected with batyl alcohol. Although the increase in platelets was not marked, thrombocytosis has been a consistent finding in all animals which received batyl alcohol. The prompt return to base-line and control values after the injections were stopped adds support to evidence favoring the existence of a specific thrombocytosis-promoting property of batyl alcohol.

The relationship of dosage to the magnitude of the erythrocytic and thrombocytic responses has been mentioned. Initially we employed a suspension of batyl alcohol in isotonic saline in doses as high as 75 mg. per day. With this preparation and dose the animals developed quite marked reactions with large areas of induration at the sites of the injections which at autopsy were found to contain material similar to that injected. The variability of the hemopoietic changes in these animals was attributed to faulty absorption. The administration of batyl alcohol in peanut oil was not associated with reactions at the injection sites or evidence of impaired absorption. However, we were unable with this vehicle to achieve a concentration greater than 6.25 mg. per milliliter, thus precluding the evaluation of large daily doses.

Studies now in progress were designed to develop and test more concentrated preparations in other media by oral as well as parenteral routes of administration in the hope that they might answer the question of the granulocytic stimulatory activity of batyl alcohol. Some preliminary observations are available and confirm the oral effectiveness of this compound. Four normal rats given a suspension of batyl alcohol via gastric intubation in doses of 50 mg. per day developed erythrocytosis, thrombocytosis, and leukocytosis. After treatment for 2 weeks, the average erythrocyte, thrombocyte, and leukocyte counts in these animals were 8,470,000 1,110,000, and 20,640 per c.mm., respectively, with base-line determinations of 6,950,000, 740,000, and 10,700. The leukocytosis was due to an increase in granulocytic elements. All values in a control group given comparable amounts of distilled water by the same route remained constant. Although the subject needs further investigation, these studies indicate that batyl alcohol does possess granulopoietic activity, but a greater amount is apparently required to evoke this response in normal rats than is needed to induce erythrocytosis or thrombocytosis.

Since batyl alcohol has been obtained from yellow bone marrow,[4, 5] it seems probable that the therapeutic results which have been attributed to yellow bone marrow and marrow extracts were due to its presence.[16, 17] Recovery of benzene poisoned leukopenic rabbits has also been reported following the intramuscular injection of the unsaponifiable fraction of yellow bone marrow.[18]

The thrombocytic and probable granulocytic stimulatory activities of batyl alcohol in addition to its action on erythropoiesis bear directly on the problem of the regulatory control of hemopoiesis. Since the erythrocytes, thrombocytes, and granulocytes have a common origin in the multipotential marrow reticulum cell, a single substance or activator-inhibitor complex may control the formation of all of these hemic elements. Batyl alcohol, originating in yellow bone marrow, may be such a substance.

SUMMARY

1. Batyl alcohol, the monoglycerol ether of n-octadecyl alcohol, is capable of stimulating erythropoiesis in normal rats. This response appears to be one of accelerated erythroblastic cellular division without associated augmentation in hemoglobin synthesis and is manifested by erythrocytosis due to microcytes with decreased osmotic resistance, reticulocytosis, and myeloid erythrocytic hyperplasia.

2. The similarities in chemical, physical, and physiologic characteristics of batyl alcohol and the thermostable, ether-soluble plasma erythropoietic factor indicate that they may be the same or closely related compounds.

3. Batyl alcohol also stimulates thrombopoiesis and probably granulopoiesis.

4. It is suggested that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms and that batyl alcohol, produced in yellow bone marrow, may be of primary importance in such a system.

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THE TYPE-SPECIFIC REACTIVITY OF THE INFLUENZA COMPLEMENT FIXATION TEST

Inability of Allantoic Fluid Antigens to Distinguish Between Asian and Other Recent Type A Influenza Virus Infections

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A LABORATORY diagnosis of influenza is generally based on serologic methods.[1, 2] In general, the techniques employed are the complement fixation test and the hemagglutination-inhibition test; the latter is much more widely utilized than the former although, from a practical diagnostic standpoint, it has several distinct disadvantages, one of which is its high degree of strain specificity in the absence of antigenic experience with related viruses within the immunologic type. This characteristic, together with the capricious and erratic behavior in hemagglutination-inhibition tests of newly isolated strains, gives rise to difficulties in laboratory diagnosis. This has been especially true with the new family of type A influenza viruses whose emergence was detected in the spring of 1957 and which are now known collectively as the "Asian" strains.

The complement fixation test, on the other hand, possesses primarily type-specific reactivities and thus is not subject to difficulties referable to marked differences in antigenic structure between the test strain and the infecting strain. That the complement fixation test may, however, exhibit some degree of strain-specific reactivity with allantoic fluid "viral" antigens has been commented upon by several workers[3-5] including ourselves.[6] Strain-specific reactivities are generally submerged in the type-specific reactions.

However, a recent report by Jensen[7] has shown that by means of complement fixation tests utilizing an allantoic fluid antigen, differences in antigenic structure of type A viruses could be detected. Thus, in human beings and ferrets infected with viruses related to the A-Denver-1-57 strain, a titer rise was seen with the homologous antigen or with an antigen made with the A-Japan-305-57 strain, but not with the A-Puerto Rico-8-34 strain.

Since Puerto Rico-34-like strains have not caused significant amounts of disease since 1947,[8] the distinction to be drawn for epidemiologic purposes is not between the PR-34 family of strains and the newly emerged Asian family

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of strains, but between the recent A-prime strains (A-Fort Monmouth-1-47-like) and these new Asian strains. Misinterpretations of Jensen's observation,[7] and certain statements of others[9] on this latter aspect have led to fairly wide dissemination of the erroneous belief that, through the use of allantoic fluids as antigens, the complement fixation test will serve to differentiate clearly between infections due to the Asian strains and the A-prime subgroup, i.e., the A-Fort Monmouth-1-47 family of type A viruses. The purpose of the present communication is to show that such a distinction is not feasible with the methods currently at hand.

MATERIALS AND METHODS

Serum Specimens Studied.—The serum specimens utilized in these studies were obtained during different years in connection with the long-term studies being conducted at Fort Ord, Calif., on viral respiratory disease. Acute and convalescent phase sera were obtained from patients who had been hospitalized with respiratory disease and had been diagnosed as suffering from influenza A on the basis of a fourfold or greater rise in complement-fixing antibody titer. Paired serum specimens from 37 patients were studied. Eight pairs of sera came from patients representing the 1953 outbreak of A-prime influenza; 10 pairs from patients representing the 1956 outbreak of A-prime influenza; and 9 pairs from patients representing the outbreak of influenza A-prime that occurred during January and February, 1957. Ten pairs of sera came from patients involved in the June, 1957, outbreak of influenza due to Asian strains of virus. The fact that the latter outbreak was due to Asian type strains was established through the isolation of viruses that were essentially indistinguishable from the A-Japan-305-57 strain.

The serum specimens came from individuals whose ages ranged from 17 to 23 years. (The patients' ages were not specifically recorded during the 1952 and 1953 outbreak but since all the men were military recruits, it is probable that the age range of the individuals studied that year was the same as that in the later years.)

All serum specimens were examined by both the complement fixation and the hemagglutination-inhibition tests.

Serologic Tests.—Hemagglutination-inhibition tests were performed essentially by the technique described by Jensen.[10] Sera were treated with potassium periodate to destroy nonspecific inhibitor.[11] One volume of serum was mixed with 2 volumes of freshly prepared M/100 potassium periodate and held overnight at 4° C.; the action of the periodate was stopped by the addition of 1.0 per cent glycerol-saline in a volume equal to the periodate added. Antigens for use in the hemagglutination-inhibition tests were prepared from the following A-prime strains: A-Berkeley-5-48 (Spirup), A-Fort Leonard Wood-1-52 (Conley), A-Albany-1-55, and A-Denver-1-57. These strains were included because they are representative of outbreaks that occurred during the years mentioned and can be considered as prototype strains. The A-Berkeley-5-48 strain was included because it is the standard strain used in this laboratory for the preparation of diagnostic complement-fixing antigens. The A-Japan-305-57 strain was used as a representative of the Asian family of viruses.

Complement fixation tests were done by the standard method employed in this laboratory over many years and recently described by Jensen.[10] Allantoic fluid antigens were prepared as follows. Eleven-day-old embryonated hens' eggs were inoculated into the allantoic cavity with an appropriately diluted suspension of virus. The eggs were incubated for 48 hours at 35° C. and then chilled overnight at 4° C. to facilitate harvesting of the allantoic fluids. Thimerosal was added to the pooled fluids in a final concentration of 1:10,000, and the fluids were distributed into tubes in 10 ml. amounts and stored at −20° C. For use, the fluids were warmed to 37° C. in a water bath and clarified by centrifugation at 1,500 r.p.m. for 5 minutes.

Complement fixation tests were done with allantoic fluid antigens prepared from the A-Berkeley-5-48 and the A-Japan-305-57 strains.

Presentation of Data.—Because of the large body of numerical data arising from the multiplicity of determinations made by the hemagglutination-inhibition test (74 sera tested against 5 antigens), the findings are presented in summary form in terms of geometric mean-fold increases between the acute and convalescent serum groups from each outbreak, as measured by each antigen.

Antibody levels as measured by the complement fixation test are presented in terms of geometric mean-fold increase in order to afford comparison with antibody levels found by the hemagglutination-inhibition test. Individual titers are also given in order to bring out more clearly the inability of the complement fixation test to determine unequivocally the influenza virus family to which the infecting virus belongs.

All titers are presented as the reciprocal of the original serum dilution. In the calculation of the geometric means, the assumption was made that titers of less than 8 had a numerical value of 4.

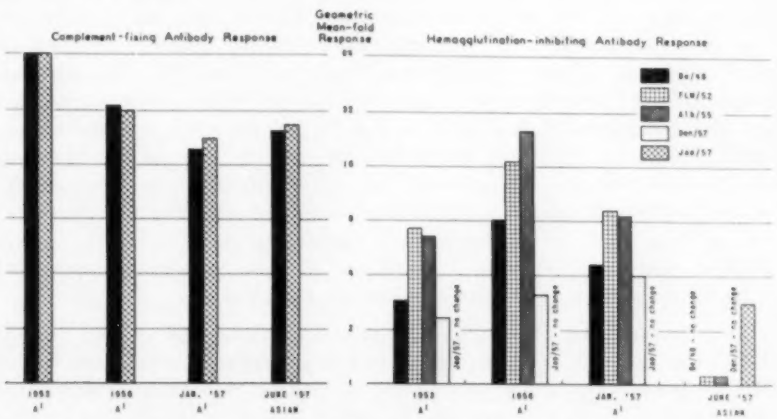

Fig. 1.—Geometric mean-fold increase in complement-fixing and hemagglutination-inhibiting antibody of patients in A-prime and Asian influenza outbreaks.

RESULTS

Hemagglutination-inhibiting Antibody.—The antibody response to infection in terms of the prototype strains is summated graphically in Fig. 1.

Considering first the paired sera from the 8 individuals involved in the 1953 outbreak, it will be seen from Fig. 1 that the geometric mean increase to the Be-48 strain was 3.1, to the FLW-52 strain it was 7.3, to the Alb-55 strain it was 6.7, and to the Den-57 strain it was 2.4. There was no demonstrable antibody or response to the Jap-57 strain.

Examination of paired sera from 10 patients with influenza during the 1956 outbreak gave qualitatively similar findings, although the titer increases were of a greater magnitude. Thus, the geometric mean-fold increase to the Be-48 strain was 8.0, to the FLW-52 strain it was 17.2, to the Alb-55 strain it was 26.0, and to the Den-57 strain it was 3.3. Again, no demonstrable antibody to the Jap-57 virus was present in the acute phase sera nor did any increases in antibody level occur.

The pattern of the antibody response during the January, 1957, outbreak was not much different from that described above except that a somewhat

smaller antibody level was encountered to all the A-prime strains except the Denver strain. The geometric mean-fold rise in antibody to the Be-48 strain was 4.7, to the FLW-52 strain it was 9.3, to the Alb-55 strain it was 8.6, and to the Den-57 strain it was 4.0. Here, too, as in the case of the 1953 and 1956 outbreaks, no antibody to the Jap-57 virus was detectable in the acute phase sera nor was a subsequent appearance of antibody to this virus strain observed.

A different pattern was seen when paired sera from 10 patients in the Asian influenza outbreak at Fort Ord in June, 1957, were examined. The hemagglutination-inhibiting antibody response to the FLW-52 and the Alb-55 strains was inconsequential; the geometric mean-fold rise in titer was only 1.15. There was no demonstrable rise in antibody to the Be-48 and the Den-57 strains. In complete contrast to the negative results obtained when the sera of individuals in the earlier outbreaks were tested against the Jap-57 strain, the geometric mean-fold increase in the antibody titer to this virus was 3.0. The low order of magnitude of these titer increases to the Jap-57 virus should be noted. It might also be mentioned that in 3 of the 10 patients there was no increase in hemagglutination-inhibiting antibody titer, and in one patient there was only a twofold increase. Unpublished data from this laboratory indicate that with the present techniques and antigens, only about one-half to two-thirds of the patients who show a diagnostic (fourfold or greater) rise in complement-fixing antibody titer show a significant increase in hemagglutination-inhibiting antibody titer when tested against the Jap-57 strain.

Complement-fixing Antibody.—Complement fixation tests using the A-Berkeley-5-48 and the A-Japan-305-57 strains as antigens were done on the acute and convalscent phase sera of all 37 patients representing the 4 outbreaks of influenza. Table I presents the titers of each of the 74 sera obtained in tests against the two antigens and Fig. 1 presents the geometric mean-fold increases in complement-fixing antibody titer to these two viruses.

As Table I shows, the complement-fixing antibody responses of the individuals studied were virtually identical whether measured against the Be-48 or the Jap-57 viruses, irrespective of the year during which the individual suffered his influenza attack. Thus, the acute and convalescent phase antibody levels of the 8 individuals representing the 1953 outbreak were identical when measured by either strain. When the sera from the 1956 outbreak were examined, antibody levels to the two viruses were identical with the exception of the fourth pair of sera which gave a twofold higher antibody titer with the Be-48 antigen than with the Jap-57 antigen. Sera obtained from patients in the January, 1957, outbreak of influenza gave identical results with both antigens in 7 instances and, in the remaining 2, a twofold greater rise in antibody was shown with the Jap-57 antigen as compared with the Be-48 antigen.

When sera obtained during the outbreak of Asian strain influenza in June, 1957, were examined, the Jap-57 antigen in 3 instances showed a twofold higher rise in antibody than did the Be-48 antigen; conversely, in one instance the Be-48 antigen showed a twofold greater increase in antibody level than did the Jap-57 antigen.

TABLE I. INABILITY OF COMPLEMENT FIXATION TEST TO DISTINGUISH INFECTIONS DUE TO ASIAN VIRUS STRAINS FROM THOSE DUE TO A-PRIME VIRUSES

RISES IN COMPLEMENT-FIXING ANTIBODY TITER DEMONSTRABLE BY			
BERKELEY-48 ANTIGEN		JAPAN-57 ANTIGEN	
ACUTE*	CONVALESCENT†	ACUTE	CONVALESCENT
Patients From 1953 Outbreak of A-Prime Influenza			
8	128	8	128
16	512	16	512
<8	256	<8	256
<8	1,024	<8	1,024
<8	1,024	<8	1,024
<8	128	<8	128
<8	512	<8	512
<8	128	<8	128
Patients From 1956 Outbreak of A-Prime Influenza			
<8	64	<8	64
<8	256	<8	256
<8	128	<8	128
16	256	32	256
<8	128	<8	128
<8	512	<8	512
8	128	8	128
<8	64	<8	64
<8	512	<8	512
<8	128	<8	128
Patients From January, 1957, Outbreak of A-Prime Influenza			
<8	256	<8	256
<8	64	<8	64
<8	64	<8	128
<8	128	<8	256
<8	64	<8	64
<8	128	<8	128
<8	16	<8	16
16	512	16	512
<8	64	<8	64
Patients From June, 1957, Outbreak of "Asian" Influenza			
<8	256	<8	512
<8	64	<8	64
<8	512	<8	512
16	128	16	256
16	128	16	128
8	64	8	64
<8	512	<8	1,024
8	64	16	64
<8	256	<8	256
8	256	8	256

*Acute = serum taken during early acute phase of illness.
†Convalescent = serum taken during convalescent stage.

It is evident from Table I that complement fixation tests with allantoic fluid antigens failed to make distinctions between viruses as markedly different as the A-prime and Asian strains. Fig. 1 further illustrates the type-specific rather than strain-specific nature of the complement-fixing antibody response. The January, 1957, influenza outbreak was due to A-prime viruses. When the Be-48 virus was used to measure antibody, the geometric mean-fold increase between the acute and convalescent phase sera was 20.2; when the Jap-57 antigen was used, the increase was 23.5. Antibody determinations on patients with Asian influenza in the June outbreak gave a geometric mean-fold increase of 26.0 when measured with the Be-48 antigen and 27.9 when measured with the Jap-57 antigen.

DISCUSSION

The data presented here and summated in Fig. 1 show rather clearly the strain-specific nature of the Asian strain hemagglutination-inhibiting antibody and the type-specific nature of the complement-fixing antibody. It is these very properties which permit detection, by the hemagglutination-inhibition technique, of antigenic differences between strains and demonstration, by the complement fixation technique, of those broad basic antigens which permit classification into immunologic types. However, although the development and existence of antibodies to the strain-specific components of the viral particle can be shown by the complement fixation method, [3-6] such antibodies are not readily demonstrable by present diagnostic techniques (cf. 6). Currently, complement-fixing antigens for diagnostic purposes consist essentially of unprocessed allantoic fluid from infected chick embryos. Such antigens comprise a mixture of the so-called "soluble" and "viral" antigens, sometimes referred to as S and V antigens, respectively. It seems probable that strain-specific reactivity, generally attributed to the V antigen, is usually overshadowed by the type-specific antigens present, and, were highly purified V antigens used, some degree of strain specificity might be achieved in the complement fixation test.

Whether such a narrow specificity would constitute a desirable feature in a test used for diagnostic purposes is debatable. Diagnostically, the type-specific reactivity of the complement fixation test is an asset since it serves to reveal similarity between strains which may differ markedly in their antigenic constitution. This is brought out in both Fig. 1 and Table I, which show that a complement-fixing antigen prepared from an A-prime virus strain could be used to measure not only the antibody response to related, yet antigenically different, A-prime viruses but, more importantly, the antibody response to a virus (Asian strain) so different antigenically from the A-prime family that it constitutes a prototype for a new family of influenza viruses. Similarly, this representative of the new family served as an excellent antigen for the detection of complement-fixing antibody not only against itself but also measurement of antibody in individuals infected by distantly related A-prime viruses. Indeed, viruses representative of either family could be used interchangeably to measure the complement-fixing antibody response called forth by infection with a member of another family.

The strain-specific reactivity of the hemagglutination-inhibition tests, illustrated here, constitutes a disadvantage from certain diagnostic points of view. Thus, to mention only one facet, failure to use current strains of virus in the test procedure may interfere with, or even preclude, detection of a virus which may be highly active in the population. This situation is illustrated in Fig. 1. It is sometimes assumed that, inasmuch as the hemagglutination-inhibition test is highly strain-specific, its application in a given instance serves to identify the infecting virus strain. This holds true under some circumstances, such as the emergence of a new family of influenza viruses, e.g., the appearance of the FM-1 family of A-prime viruses in 1947 and the emergence of the Asian strain

family in 1957. Specific identification of these agents as the infecting virus in a given instance was feasible only because they were so different antigenically from previous strains that the population possessed no demonstrable antibody to them. With time, however, the new viruses become so thoroughly and repeatedly seeded throughout the population that infection or vaccination subsequently leads to a recall of antibody to those viral strains with which the individual has had extensive prior experience, i.e., infection or artificial immunization leads to a humoral antibody response that recapitulates the individual's previous experience with influenza virus antigens.[6, 12-15]

SUMMARY

The influenzal complement-fixing antigens in general use for diagnostic purposes possess marked type-specific reactivities. Hence, while the complement fixation test serves effectively to reveal the immunologic *type* of the virus strain which produced infection in the patient, it cannot be expected to reveal the *family* within that type to which the virus belongs. To determine this, the patient's sera should be examined by the hemagglutination-inhibition test, which is characterized by strain-specific reactivities.

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CLINICAL USEFULNESS OF CONSECUTIVE FOUR-DAY I^{131} SERUM LEVELS IN THYROID DISEASE

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IN OBSCURE cases, or where prior therapy has clouded the clinical status, Means[1] has noted that the use of all available tests can aid in evaluating thyroid function. We have found that it is particularly helpful to perform consecutive daily analyses of the total and protein-bound iodine^{131} in the serum, and to study the curves constructed from these data.

From single determinations of total serum I^{131} and PBI^{131}, the conversion ratio was first proposed by Clark and associates[2] in an attempt to separate the various thyroid states. In a later publication, Silver and co-workers[3] determined the I^{131} level of whole blood up to 4 days and included single 72-hour PBI^{131} plasma determinations.[4] These authors found a wide range in the hyperthyroid group, with overlapping into the euthyroid range. Scott and Reilly[5a] and others[5b, 5c] have noted the differences in PBI^{131} plasma values up to 96 hours in patients with varying thyroid function using resins for the separation of inorganic from organic I^{131} in plasma.

According to Rosenthal and Lawrence,[6] recent reports have been concerned with erratic results of PBI^{131} values obtained from patients following previous surgical or I^{131} treatment of hyperthyroidism. Individual determinations of PBI^{127} have been widely accepted as a most accurate measure of thyroid activity. However, recent studies of Margolese and Golub[7] note the daily fluctuations of the serum PBI level in 2 individuals. According to Barrett and associates[8] a single serum PBI^{127} is not helpful in diagnosis of hypothyroidism.

The purpose of this study is to record more accurately the functioning of the thyroid gland by studying the dynamic distribution of I^{131} in the serum over varying periods of time.

From hourly and daily consecutive 10-day determinations, we have selected the 4-day consecutive levels of total serum I^{131} and the conversion ratio of organic-bound I^{131} to the total I^{131} in serum. Curves from these data supplied the most representative reflections of hormone production and clinical status of the human thyroid gland in the patients examined.

METHODS

The tracer dose used was 75 μc of carrier-free I^{131} orally administered. Blood was collected at 24-hour intervals and the serum separated. The I^{131} activity of 4 ml. of

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serum was determined in a scintillation counter. The serum was then passed over a column (1 cm. in diameter) containing 500 mg. Dowex 2 resin in the chloride form. The resin was washed twice after filtering with 1 ml. portions of saline. The protein of the filtrate was concentrated by precipitation with 20 per cent trichloroacetic acid in order to adjust the volume to 4 ml., suitable to the well of the scintillation counter. The protein precipitate was resuspended in the necessary amount of saline and the radioactivity determined.

The conversion ratio is defined as the ratio $\frac{\text{counts organic-bound } I^{131}}{\text{total counts } I^{131}}$. Total counts were extrapolated to 1 ml. and the values plotted on 1 cm. square graph paper. No correction was made for decay since an easily reproducible method was desired. The per cent dose per liter or actual values were plotted against time in days. The standard deviation from the mean for each daily time value has been calculated.

The thyroid status of the individuals studied, all of whom were patients in Veterans Administration Hospitals, was determined from clinical examinations which included medical history, physical examination, 6- and 24-hour I^{131} thyroid uptakes, and I^{131} urinary excretions, as well as basal metabolic rate and serum cholesterol determinations.

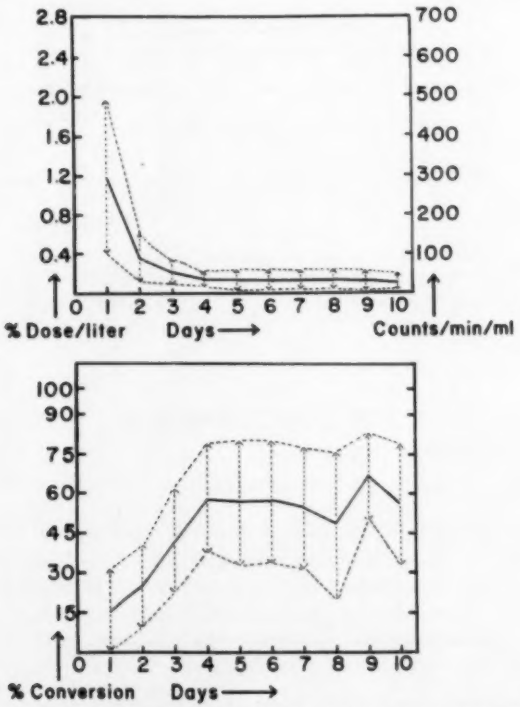

Fig. 1*A*.—The upper curve represents per cent dose per liter of total serum I^{131} in 18 euthyroid patients under 60 years of age. The lower curve denotes per cent conversion. Both are for a 10-day period. In this and subsequent figures, the continuous line represents the mean, and the broken lines represent the standard deviation.

RESULTS

Euthyroid Group.—The results obtained on a group of euthyroid patients are presented in Figs. 1*A* and 1*B*. Serial daily total serum I^{131} determinations

and conversion ratios were obtained from a group of 41 euthyroid males of varying ages. The curves were plotted to 10 days, and it was noted that they leveled out after 4 days. The 4-day curves, Fig. 2, *A*, represent the basic normal criteria used for comparison with the curves plotted from data obtained from subsequent groups of patients to be presented.

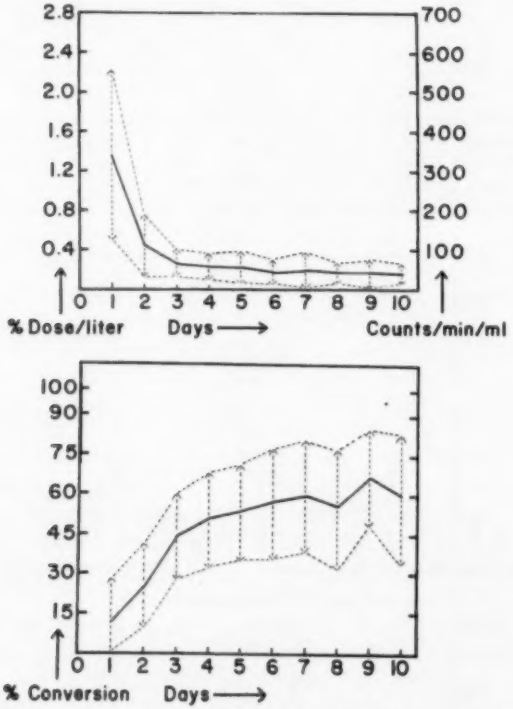

Fig. 1*B*.—The same as for Fig. 1*A*, except for 25 patients over 60 years of age.

Hypothyroid Group.—Data from a group of hypothyroid patients are presented in Fig. 2, *B*. The initial serum level of total I^{131} at 24 hours was higher and the rate of fall was steeper in this hypothyroid group than in the basic euthyroid group. In analyzing the data for this group, it was noted that the 24-hour value representing the conversion ratio is lower and the curve is continuously flatter than the curve representing the basic euthyroid group.

Hyperthyroid Group.—Curves from data from a group of hyperthyroid patients are presented in Fig. 2, *C*. At the 24-hour level the value for total serum I^{131} was lower than in the euthyroid or hypothyroid groups, rose slightly at the 48-hour period, and leveled off thereafter. The conversion ratios were much higher for the hyperthyroid patients at the 24- and 48-hour periods, but there was a leveling off after 72 hours.

Euthyroid Group After Surgery.—The data from a group of hyperthyroid patients who became euthyroid after surgery are presented in Fig. 3, *A*. The total serum I^{131} curve resembled the curve for the euthyroid group. In contrast, the conversion ratio value was somewhat higher than that representing the basic euthyroid group at the 24-hour period and leveled off after 48 hours, resembling the conversion ratio curve for the hyperthyroid group.

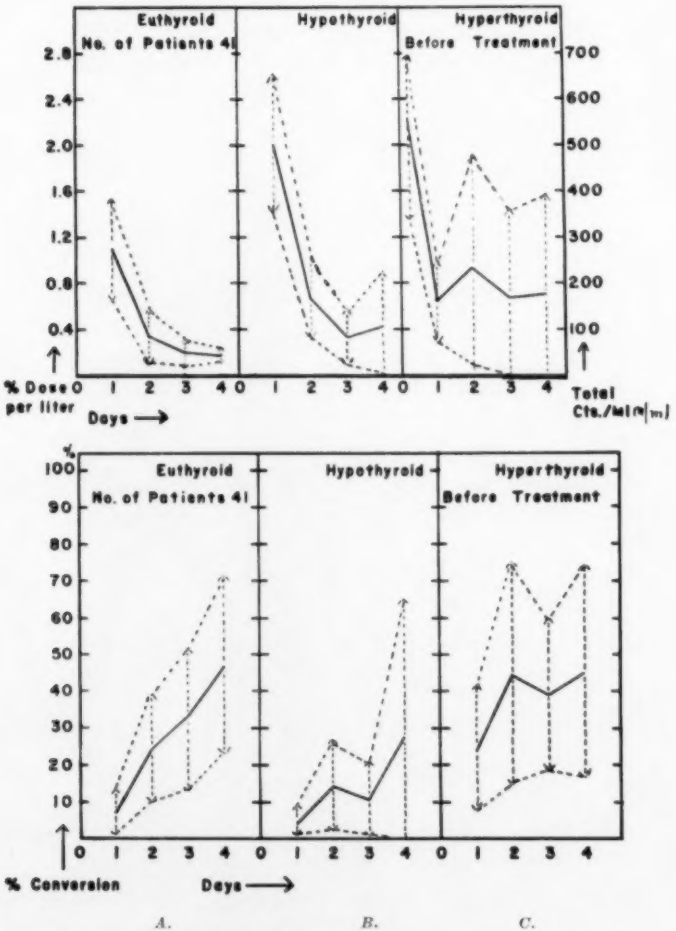

Fig. 2.—Per cent dose per liter and conversion curves up to 4 days for (*A*) euthyroid, (*B*) hypothyroid, and (*C*) hyperthyroid groups of patients.

Euthyroid Group After I^{131} Treatment.—A hyperthyroid group of patients, who became euthyroid following treatment with I^{131}, were studied next, and

data from this group are noted in the curves presented in Fig. 3, *B*. The total serum I^{131} curve was similar to the basic euthyroid group curve, but, like the surgically treated patients, the conversion ratio at the 24-hour period was higher than that in the basic euthyroid group at a similar point of time. The entire contour of the conversion ratio curve for this group resembled the hyperthyroid type of curve.

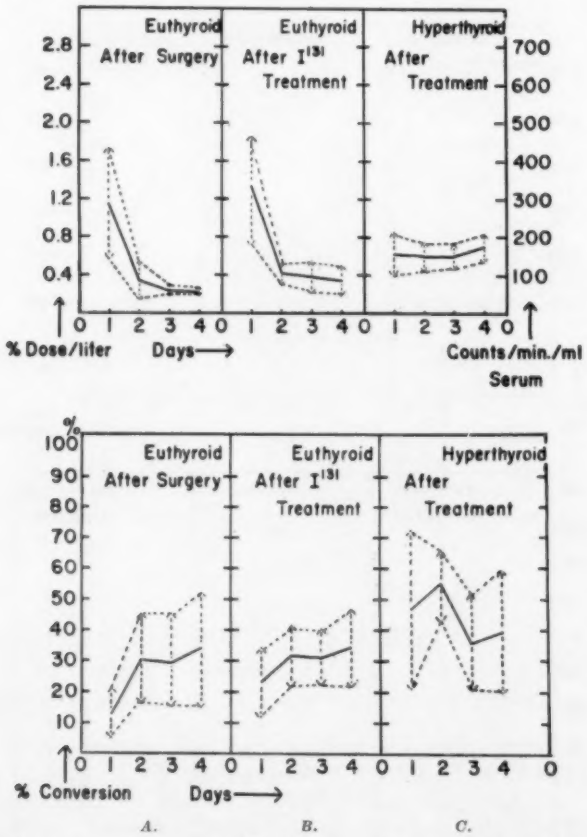

Fig. 3.—Curves representing groups of hyperthyroid patients treated by surgery (*A*) or with I^{131} (*B*), and resulting in a euthyroid state following treatment. Curves from a group of patients remaining hyperthyroid after surgery or treatment with I^{131} (*C*).

Hyperthyroidism Persistent After Treatment.—Patients who remained hyperthyroid despite surgery and I^{131} treatment constituted the next group. As noted in Fig. 3, *C*, both curves resembled those for the hyperthyroid patients before treatment as shown in Fig. 2, *C*.

Individual Illustrative Cases.—The data presented in Fig. 4, *A* through *D*, were obtained from a 28-year-old white man with the clinical symptoms of

hyperthyroidism. The original data from which the curves were plotted were obtained in October, 1956, and correlated in all respects with the curves for severe hyperthyroidism.

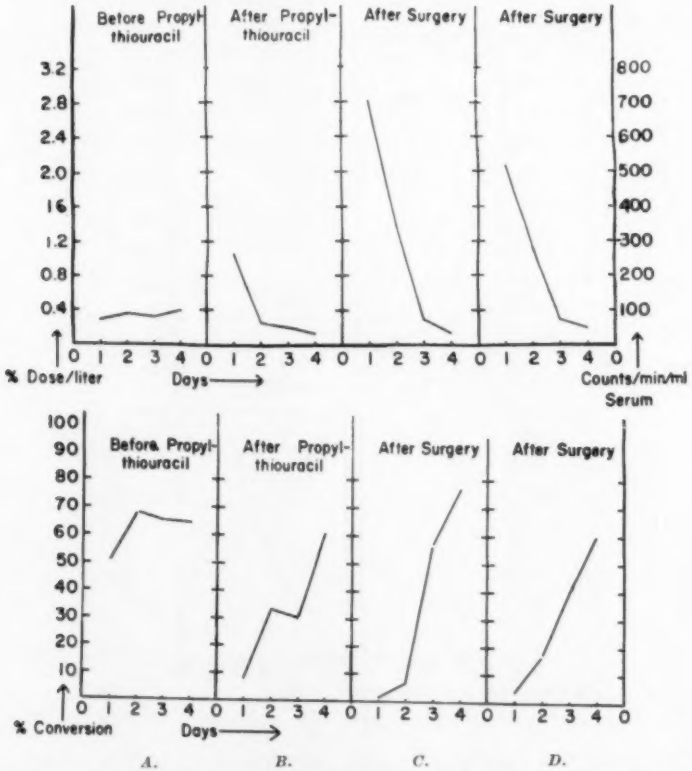

Fig. 4.—Curves from a hyperthyroid patient, before (*A*) and following (*B*) propylthiouracil therapy and 15 weeks (*C*) and 20 weeks (*D*) following subtotal thyroidectomy. The curves before surgery are similar to the curves for the hyperthyroid group in Fig. 2, *C*, and those following surgery are similar to the curves for the hypothyroid group in Fig. 2, *B*.

Following a course of propylthiouracil, which consisted of a 100 mg. dose of the drug 3 times daily for 12 weeks, the total serum I^{131} and conversion ratio curves returned to a euthyroid configuration, as noted in Fig. 4, *B*.

A subtotal thyroidectomy was performed on this patient on Feb. 12, 1957, and a recheck of the total serum I^{131} and conversion ratio curves from data obtained 15 weeks after surgery is demonstrated in Fig. 4, *C* and conform to the typical curves of the hypothyroid group depicted in Fig. 2, *C*.

An examination was repeated 5 months later, as portrayed by the curves in Fig. 4, *D*. The conformation of these curves still resembled those of the hypothyroid group, though they show signs of reversion to the basic euthyroid type of curve.

The data from which the curves in Fig. 5, *A* and *B*, were constructed were obtained from a 70-year-old white man suffering with chronic, progressive post-encephalitic Parkinson's disease with senile deterioration. Originally, the total serum I^{131} and conversion ratio curves resembled the hypothyroid group curves presented in Fig. 2, *B*.

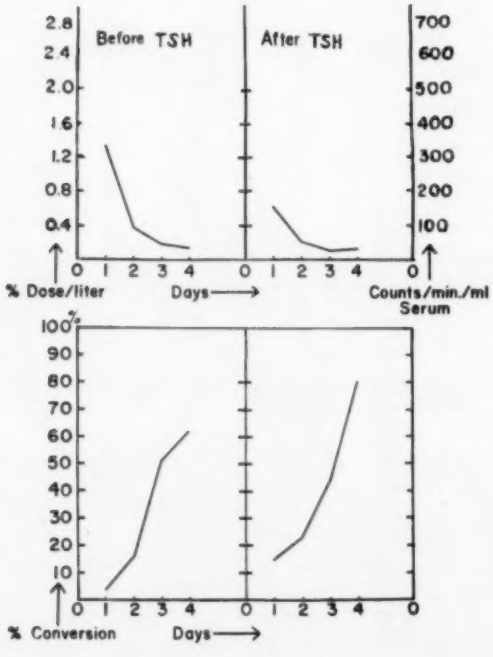

Fig. 5.—Patient whose original curves (*A*) resemble those for hypothyroidism as shown in Fig. 2, *B*. Following test dose of 10 units of THS, the curves (*B*) resemble euthyroid group configuration as in Fig. 2, *A*.

Data for the curves noted in Fig. 5, *B* were obtained 48 hours following the administration of 10 units of thyroid stimulating hormone (TSH). The trend of the curves following TSH is toward the euthyroid configuration and would indicate a functioning thyroid gland and secondary hypothyroidism due to hypothalmic or pituitary disease.

A 57-year-old white man who had originally suffered from hyperthyroidism, but who had been athyrotic following surgery and I^{131} therapy, afforded data for the curves in Fig. 6, *A* and *B*. Following TSH administration, the curves demonstrated no change, nor was the I^{131} thyroid uptake increased. These results indicate that in this patient there is lack of functioning thyroid tissue.

The curves shown in Fig. 7 represent data from a 71-year-old white man who had been on previous doses of potassium iodide. The total serum I^{131} value

at the 24-hour interval was extremely elevated and the curve fell off rapidly from that level. The conversion ratio curve was unique, in that it had a downward slope to points no longer detectable. These curves are typical of the saturation of the thyroid gland with potassium iodide.

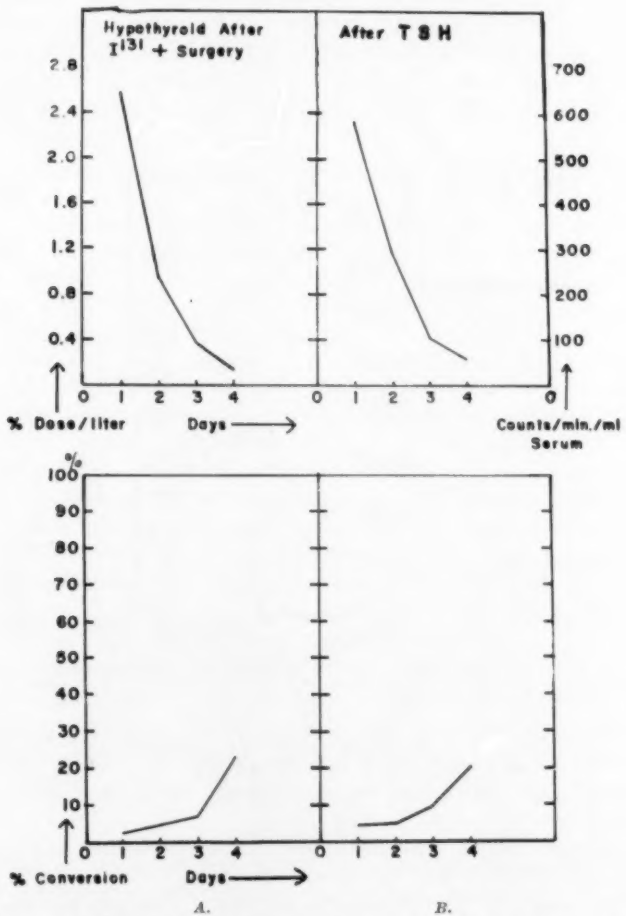

Fig. 6.—*A*, Curves from a patient, originally hyperthyroid, who had been previously treated both by subtotal thyroidectomy and subsequently with I^{131}. *B*, Following test dose of 10 units of TSH, the curves remained similar to the hypothyroid group type, Fig. 2, *B*, indicating lack of functioning thyroid tissue.

DISCUSSION

At the inception of these studies, it was originally intended to measure the total I^{127} in the serum in addition to performing radioactive determinations. It was soon found that the values of I^{127} in the serum showed little difference in

the bloods from patients whose thyroid metabolic status ranged from hypothyroid to hyperthyroid. This narrow range, when measured by total I^{127} values, does not afford a sufficient differentiation of the patient's thyroid status. In addition, the minute quantities of I^{127} in the serum are difficult to determine. The avidity of the thyroid gland for iodine leaves little excess for circulation in the serum. For example, following ingestion of a test dose of I^{131}, the amount of total I^{131} found in serum after the fourth day is 0.16 per cent per liter. The remainder has either been taken up by the thyroid gland (approximately 30 per cent), or excreted (approximately 60 per cent), or diffused into extracellular fluid.

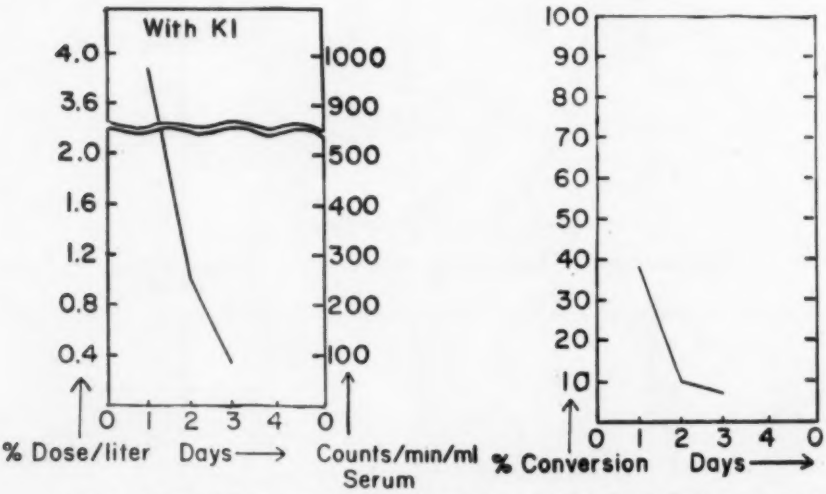

Fig. 7.—Curves obtained from a patient following previous ingestion of a medication containing potassium iodide. Downward slope of conversion ratio curve is diagnostic of previous iodine uptake by the thyroid gland.

When the alkaline ashing method of Barker[9] was used, 50 per cent of added radioactive iodine was recovered at 650° C. ashing temperature. Using lower temperatures it was found that the radioactivity was retained, but the ashing was not complete.

Kolthoff and Jordan[10] recommended the polarographic determination of minute amounts of iodine with the rotating platinum electrode, but in our experience it was found that the method was not sensitive enough at concentrations below 10^{-3} molar. The colorimetric method, as described by Sandell and Kolthoff,[11] was satisfactory from the analytic point of view, but very time consuming. The preparation of standard curves in each instance makes it impracticable. The amount of stable iodine in the hypothyroid state is often no greater than the amount in the blank determination, so that the accuracy of the determination suffers. Because of the difficulty of the method and its inaccuracy at minimal levels, as compared with results obtained by using I^{131}, we have not included the stable iodine determinations in our data.

It has been found that the absolute amount of protein-bound iodine^{131} in the hyperthyroid patient is usually not more than double the level in the euthyroid patient. But, if the rate of conversion to organic iodine, as reflected by the conversion rate curve, is noted, the distinction between these 2 types of patients is much more apparent and the differentiation more easily made.

Rienhoff[12] and Roussy and co-workers[13] investigated the duration of thyrotoxicosis in the gland fragment remaining after surgery, using histologic hyperplasia as a criterion. Rienhoff studied the glands of 6 previously hyperthyroid patients coming to biopsy up to 6 years after operation, and found persistence of hyperplasia in all.

In patients rendered euthyroid after treatment by surgery or by I^{131}, the conversion ratio curves were similar to the curves of hyperthyroid patients before treatment. In all probability, this is so because the gland remnant is turning over at the same rate as was the whole hyperthyroid gland before treatment. Nevertheless, the reduction in quantity of iodine retained by the gland remnant is evidenced by the total I^{131} serum curves, which now resemble the euthyroid rather than the hyperthyroid type of curve. The continued increased metabolism of the thyroid gland remnant after treatment is thus established. These results have been misinterpreted as "false positive" results by a number of observers. Paley and associates[14] reported a higher proportion of "false positive" results in patients who had previous surgery and iodine^{131} therapy than in untreated patients. Likewise, Silver and co-workers,[15] in an article on pitfalls in the diagnostic use of radioactive iodine, have based their conclusions on single protein-bound I^{131} determinations from bloods drawn 72 hours after the administration of the tracer dose. Had they calculated daily conversion ratios, they probably would not have been misled by the data obtained from their patients.

We have calculated the standard deviation from the mean for the data here presented at each daily time interval. There is a wide range with overlap in the different conditions studied. This is unavoidable when differentiation into only 3 groups is attempted. Nevertheless, the slope of the curves obtained from the 3 groups of patients is so characteristic, that when both total serum I^{131} and conversion ratio curves are analyzed together, a definite differentiation of the patient's status can be made.

SUMMARY

1. Total I^{131} in serum was determined, and resin separation of I^{131} into inorganic and organic fractions in the serum was carried out. A consecutive daily survey was performed on patients with varying thyroid metabolic activity. Six and 24-hour I^{131} thyroid uptake and total 24- and 48-hour urinary excretion tests were performed on all patients studied, in addition to other clinical and laboratory tests of thyroid function.

2. Inspection of the curves from the data obtained showed characteristic slopes of ascent and descent depending on the clinical status of the patient. At any particular time interval, the direction of the curve for the various conditions studied may be different; nevertheless, absolute values can be approximated.

From this it is concluded that single determinations of total serum I^{131}, or protein-bound I^{131}, may lead to serious misinterpretations of the metabolic thyroid state of the patient under study.

3. In studying the results of daily serial total serum I^{131} and conversion ratio curves on a group of patients, we have noted a leveling off after the fourth day following the ingestion of the test dose. This is demonstrated in our curves for euthyroid patients.

4. We have presented 4-day curves for determinations of total serum I^{131} and conversion ratios for a group of euthyroid subjects of various ages. These curves represent basic normal criteria for the study of dynamic distribution of I^{131} in the blood. They were used as standards for comparison with curves from hypothyroid and hyperthyroid subjects and individual variants.

5. Curves for subjects formerly hyperthyroid, who had been rendered euthyroid after treatment with surgery as well as after treatment with radioactive I^{131}, have been presented and it was noted that the total serum I^{131} curves reverted to the type representing the euthyroid, while the conversion ratio curves continued to resemble their original shape characteristic of hyperthyroidism. This confirms the fact that there is continued increased metabolism of the thyroid gland remnant after both types of treatment.

6. In contrast, curves from groups of subjects remaining hyperthyroid following surgery and after radioactive I^{131} treatment, retain the contour of the hyperthyroid patient.

7. Individual curves illustrating partial hypothyroidism following surgery, athyreosis, secondary hypothyroidism as differentiated by the administration of TSH, and the effect of previous iodine administration are all presented and analyzed as illustrative cases.

8. Our experience with the determination of minute amounts of I^{127} in the serum are presented.

9. It is concluded that total blood serum I^{131} and conversion ratio curves here described and consecutively determined for 4 days allowed evaluation of the status of the individual patients and the metabolic state of their thyroid gland.

10. The curves presented offer a practical and sensitive method for the evaluation of the clinical thyroid status of the individual patient, particularly in obscure cases, or where prior therapy has clouded the clinical status and other laboratory findings.

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EVIDENCE THAT GLASS INCREASES PLASMA PTA ACTIVITY

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SOLVEIG MIKKELSEN

A GLASS surface initiates coagulation not by activating platelets[1] but by activating plasma clotting factors. Glass contact has been shown to increase both PTC (plasma thromboplastin component, antihemophilic B factor, Christmas factor)[2, 3] and proconvertin (factor VII, stable factor)[3] activity. However, Shafrir and DeVries[1] also demonstrated glass-enhanced clotting activity in barium sulfate adsorbed hemophilia A (AHG deficient) plasma. Since such adsorbed plasma contains no AHG, PTC, or proconvertin, these authors postulated activation of an additional plasma thromboplastic factor, either PTA (plasma thromboplastic antecedent) or Hageman factor. The following data demonstrate that glass contact does increase plasma PTA activity.

MATERIALS AND METHODS

Reagents.—

1. *PTA deficient "glass-activated" substrate plasma:* PTA deficient plasma was obtained from the 3 patients with PTA deficiency described below. Nine volumes of venous blood was added to one volume of 0.1 M sodium citrate and centrifuged at 10,000 r.p.m. The resultant platelet-poor plasma was poured into a 12 by 125 mm. glass tube containing two 3 mm. glass beads per milliliter of plasma. The plasma was shaken by hand for 10 minutes at room temperature. It was corked and stored in a glass tube at 4° C. for approximately 4 hours until used as PTA deficient glass-activated substrate plasma.

2. *Test plasmas:* a. Normal Silicone and Glass-Activated Plasmas. Nine volumes of normal venous blood, obtained with a needle coated with Monocote E (The Armour Laboratories) and a silicone-coated syringe (G.E. SC-87, Dri-film), was added to one volume of 2 per cent sodium oxalate in a chilled, silicone-coated tube and immediately centrifuged at approximately 4,000 r.p.m. The resultant platelet-poor plasma was divided into 3 portions. One was transferred with a silicone-coated pipette to a second silicone-coated tube, corked, and stored at 4° C. until used as normal silicone plasma. A second portion was transferred to a glass tube and activated with glass beads as described above. This was corked and stored in a glass tube for use as normal glass-activated plasma.

b. Adsorbed Normal Silicone and Glass-Activated Plasmas. The third portion of the silicone plasma referred to above was transferred with a silicone-coated pipette into a silicone-coated tube to which was added 75 mg. of barium sulfate (Baker) per milliliter of plasma. After frequent shaking for 15 minutes at room temperature, the barium sulfate was removed by

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centrifugation. Half of this adsorbed plasma was stored in a corked silicone-coated tube at 4° C. until used as adsorbed normal silicone plasma. A second portion was transferred to a glass tube, activated with glass beads and stored as described above until used as adsorbed normal glass-activated plasma.

All test plasma were used within about 4 hours after the blood was drawn. Dilutions were made in a fluid containing 1 part of 2 per cent sodium oxalate and 5 parts of 0.9 Gm. per cent sodium chloride. This prevented variation in the oxalate content as a result of dilution.

3. *Cephalin:* Cephalin, an acetone insoluble, ether soluble fraction of human brain, was prepared and diluted in veronal buffer as described elsewhere.[4] Normal washed platelets were used instead of cephalin in one experiment. The platelets from 17 ml. of normal blood were washed 3 times in about 10 ml. of a fluid made up of 2 parts of veronal buffer, 2 parts of 25.66 mM. sodium citrate, and 6 parts of 0.9 Gm. per cent sodium chloride. The platelets were resuspended in 6 ml. of the same fluid to give a concentration of about 404,000 per cubic millimeter.

Methods.—

1. *To demonstrate glass activation of PTA:* The use of a modified partial thromboplastin time test to demonstrate glass activation of a plasma thromboplastic factor has been discussed in detail in an earlier publication.[3] The same technique was employed in this study except that the substrate plasma was PTA deficient. The clotting mixture consisted of: 0.2 ml. of glass-activated PTA-deficient substrate plasma (providing a relative excess in glass-activated form of every plasma clotting factor except PTA); 0.2 ml. of a dilution of the test plasma, either silicone or glass-activated normal or normal adsorbed plasma (providing the only source of PTA activity for the clotting mixture); 0.2 ml. of cephalin diluted 1:50 in veronal buffer (providing an optimal amount of platelet thromboplastic factor-like activity); and, after 3 minutes' incubation at 37° C., 0.2 ml. of 35 mM. calcium chloride.

When silicone plasma was being tested, silicone-coated glassware was used throughout.

Since this mixture provides an optimum amount, in glass-activated form, of all other clotting factors, the PTA activity of the test plasma determines its clotting time. Therefore, at any dilution tested, a shorter clotting time obtained with glass activated than with silicone test plasma indicates more PTA activity in the former.

2. *To prove the substrate plasma PTA deficient:* Partial thromboplastin time and thromboplastin generation tests were used in proving that the 3 patients supplying the substrate plasma were PTA deficient. The partial thromboplastin time was done as described above, except that the patient's plasma and the "correcting reagents," prepared in glass tubes, were not activated with glass beads. A 1:5 dilution of the correcting reagents was used.

For the thromboplastin generation test, the incubation mixture consisted of equal parts of cephalin suspension (in place of platelets), of adsorbed patient's plasma, of patient's serum, of a correcting reagent, and of 30 mM. calcium chloride. The cephalin was diluted 1:50 in veronal buffer. Oxalated plasma was adsorbed with barium sulfate, and citrated plasma with aluminum hydroxide.* Adsorbed plasma was diluted 1:5 in buffer before use. Serum was incubated on the clot for 2 hours at 37° C., citrated with one volume of 0.1 M sodium citrate for each 9 volumes of blood, and incubated for 1 hour more at 37° C. Serum was diluted 1:10 in buffer before use. The correcting reagents, some of which had been stored in the liquid frozen state, were diluted 1:5 if plasma and 1:10 if serum.

At 3-minute intervals after the mixture was recalcified, 0.2 ml. of the mixture and 0.2 ml. of 20 mM. calcium chloride were added to 0.2 ml. of substrate plasma. The substrate plasma was either the patient's or normal plasma.

Quantitative AHG and PTC assays were also performed. Pool's[5] AHG assay technique was used. The PTC assay was based upon the correction of the abnormal thromboplastin generation found with glass-activated PTC deficient serum by a 1:100 dilution of glass-activated test serum, and will be described in detail in a future publication.

*Suspension No. 7371, Cutter Laboratories, Berkeley, Calif.

RESULTS

1. *Proof That the Substrate Plasma Lacked PTA Activity.*—The experiments on glass activation are based upon shortening the long partial thromboplastin time of a PTA deficient substrate plasma. Therefore, they require proof, provided below, that the patients furnishing this substrate plasma fulfilled the criteria for PTA deficiency.[6, 7]

CASE 1.—J. L., a 41-year-old policeman, neither bruises easily nor bleeds unusually from small cuts. He played high school football without trouble. However, he bled for about a week following a tonsillectomy, and also following 2 tooth extractions. A large, incisional hematoma formed after a herniorrhaphy. His parents, brother and sister, and son and daughter were said to be normal. Our laboratory studies of his son and daughter were normal.

This patient's blood has been examined repeatedly over a 2-year period. Lee-White clotting times in glass were not prolonged. He formed a firm clot of normal size, which retracted promptly and did not lyse. His bleeding time, platelet count, Quick prothrombin time, prothrombin-proconvertin test,[8] Russell's viper venom-cephalin prothrombin test,[9] and one-stage proaccelerin assay were all normal.

TABLE I. PARTIAL THROMBOPLASTIN TIMES ON PATIENT J. L. (CASE 1)

CORRECTING REAGENT	PARTIAL THROMBOPLASTIN TIME* (NORM. 50-90 SEC.)
Unadsorbed Plasma	
Patient J. L., fresh	141
Patient J. L., stored	142
Normal, fresh	98
Hemophilia A, stored	85
Hemophilia B, stored	93
$Al(OH)_3$ Adsorbed Plasma	
Normal, fresh	99
Hemophilia A, stored	111
Hemophilia B, stored	94
Unadsorbed Serum	
Normal, fresh	80
Hemophilia B, stored	84
Hageman deficiency, stored	45
Patient J. L., stored	66
Patient E. G. (Case 3), stored	75
$Al(OH)_3$ Adsorbed Serum	
Normal, fresh	102
Hemophilia B, stored	92

*System: 0.2 ml. of patient's undiluted plasma; 0.2 ml. cephalin 1:50; 0.2 ml. correcting reagent 1:5; 0.2 ml. 35 mM.$CaCl_2$. The times listed are averages of two determinations.

Prothrombin consumption, partial thromboplastin time, and thromboplastin generation tests have consistently demonstrated impaired intrinsic thromboplastin formation. Representative partial thromboplastin time values are listed in Table I. They show that normal, AHG deficient (hemophilia A), and PTC deficient (hemophilia B) plasma shortened the prolonged partial thromboplastin time of his fresh plasma, whereas his own plasma, stored liquid

frozen for several weeks, did not. Shortening persisted after aluminum hydroxide adsorption but was not quite as complete. This is expected since adsorption partly removes PTA activity.[7]

Table I also shows that normal serum, stored PTC deficient serum, and stored Hageman factor deficient serum completely corrected, and adsorbed normal and PTC deficient serum partly corrected, his long partial thromboplastin time. However, these data must be interpreted with the knowledge that his own serum, stored frozen for 11 months, and serum from Patient E. G. (Case 3), stored frozen for 3 months, also corrected his clotting time. Rosenthal[7] and Laurrieu and her co-workers[10] have described increased PTA activity on storage that may interfere with matching studies using frozen samples.

Table II lists the shortest times obtained in a thromboplastin generation test when various correcting reagents were added to the incubation mixture. It shows that adsorbed normal plasma, adsorbed normal plasma treated with thrombin (to destroy AHG), adsorbed hemophilia A plasma, normal serum, and hemophilia B serum all corrected his defective thromboplastin generation. Oxalated normal serum adsorbed with barium sulfate did not completely correct his thromboplastin generation test.

TABLE II. SHORTEST THROMBOPLASTIN GENERATION TEST TIMES ON PATIENT J. L. (CASE 1)

CORRECTING REAGENT	CLOTTING TIME (NORM. 9-13 SEC.)
Buffer	18
$BaSO_4$ Adsorbed Plasma	
Normal	13
Normal, thrombin treated	10
Hemophilia A	12
Serum	
Normal	9.5
Hemophilia B	9.5
Normal, $BaSO_4$ adsorbed	15.5

The patient's plasma corrected the abnormal thromboplastin generation test of a patient with hemophilia A, and his serum corrected the abnormal thromboplastin generation of several patients with hemophilia B. Quantitative assay of his plasma for AHG and his serum for PTC yielded values of 96 and 160 per cent, respectively.

CASE 2.—D. G. is a 27-year-old housewife without unusual bruising. Her menstrual periods last 2 weeks, with one week of heavy flow. She escaped serious bleeding after a tonsillectomy, a tooth extraction, and 2 deliveries. However, she bled for 2 weeks following extraction of a wisdom tooth. A severe hemorrhage, requiring transfusions, followed a cervical biopsy. Studies at that time by Dr. Maurice Yettra of the Southern California Permanente Medical Group led to a tentative diagnosis of PTA deficiency. Her father and a paternal aunt are said to bleed following surgery or tooth extraction.

We found that her blood clotted without delay in glass tubes, forming a firm clot of normal size which retracted promptly and did not lyse. Her platelets were adequate in number and appearance on stained smear. Her Quick prothrombin time, prothrombin-proconvertin test, Russell's viper venom-cephalin prothrombin test, one-stage proaccelerin assay, and thrombin time were all normal.

Prothrombin consumption, partial thromboplastin time, and thromboplastin generation tests disclosed impaired intrinsic thromboplastin formation. The effect of various correcting reagents upon her partial thromboplastin and her shortest thromboplastin generation test time is shown in Table III. These data show that adsorbed normal, hemophilia A, and hemophilia B plasmas shortened her partial thromboplastin time significantly and to the same extent, whereas adsorbed plasma from Case 1 (J. L.) did not. Normal and hemophilia B serum completely corrected her partial thromboplastin time, and contrasted with stored serum from Case 1 which gave incomplete correction. Partial correction persisted after aluminum hydroxide adsorption of citrated normal and hemophilia B serum.

TABLE III. PARTIAL THROMBOPLASTIN TIMES AND SHORTEST THROMBOPLASTIN GENERATION TEST TIMES ON PATIENT D. G. (CASE 2)

CORRECTING REAGENT	PARTIAL THROMBOPLASTIN TIME* (NORM. 50-90 SEC.)	THROMBOPLASTIN GENERATION TIME (NORM. 9-13 SEC.)
Buffer	—	18
Unadsorbed plasma		
Patient D. G., fresh	145	—
Normal, fresh	72	—
$Al(OH)_3$ adsorbed plasma		
Normal, fresh	107	10
Hemophilia A, stored	106	—
Hemophilia B, stored	106	—
Patient J. L. (Case 1), stored	135	—
Unadsorbed serum		
Normal, fresh	75	9.0
Hemophilia B, stored	83	11.5
Patient J. L. (Case 2), stored	110	—
$Al(OH)_3$ adsorbed serum		
Normal, fresh	95	—
Hemophilia B, stored	103	—

*Averages of two determinations.

Table III also shows that normal thromboplastin generation test times were obtained when normal adsorbed plasma, normal serum, or hemophilia B serum was added to the incubation mixture. Quantitative AHG assay of her plasma and PTC assay of her serum gave values of 90 and 70 per cent, respectively.

CASE 3.—E. G. is a 41-year-old white woman who "bled for days" after a tonsillectomy and after each of 4 tooth extractions. She bled excessively after one delivery but not after another. Hemorrhage following a nasal polypectomy was severe enough to require a transfusion. Her menstrual periods have always been heavy. Spontaneous ecchymoses have been rare and small, and bleeding from cuts has always stopped without difficulty.

She stated that her mother also bled easily and had required 17 units of blood during a radical neck dissection. Her father, brother, and 2 sons have not bled abnormally.

Her blood clotted promptly in glass tubes, and her plasma behaved normally in the Quick prothrombin, prothrombin-proconvertin, Russell's viper

venom-cephalin prothrombin, one-stage proaccelerin, and thrombin time tests. Partial thromboplastin time, prothrombin consumption, and thromboplastin generation tests revealed impaired intrinsic thromboplastin formation. As Table IV shows, this was corrected by normal plasma, adsorbed normal plasma, adsorbed hemophilia A plasma, normal serum, hemophilia B serum, and Hageman factor deficient serum. In contrast, stored adsorbed plasma from Case 1 (J. L.) failed to correct either her partial thromboplastin time or her thromboplastin generation test. However, his stored serum partly corrected her partial thromboplastin time and completely corrected her thromboplastin generation test. Quantitative AHG assay of the patient's plasma and PTC assay of her serum yielded values of 80 and 120 per cent, respectively.

TABLE IV. PARTIAL THROMBOPLASTIN TIMES AND SHORTEST THROMBOPLASTIN GENERATION TEST TIMES ON PATIENT E. G. (CASE 3)

CORRECTING REAGENT	PARTIAL THROMBOPLASTIN TIME* (NORM. 50-90 SEC.)	THROMBOPLASTIN GENERATION TIME (NORM. 9-13 SEC.)
Buffer	240	19.5
Unadsorbed Plasma		
Normal, fresh	88	—
$BaSO_4$ Adsorbed Plasma		
Normal, fresh	100	11.5
$Al(OH)_3$ Adsorbed Plasma		
Normal, fresh	95	—
Hemophilia A, stored	101	14.5
Patient J. L. (Case 1), stored	185	18.0
Unadsorbed Serum		
Normal, fresh	74	10.0
Hemophilia B, stored	77	12.5
Hageman deficiency, stored	59	13.0
Patient J. L. (Case 1), stored	125	11.0

*Averages of two determinations.

2. *Proof That Glass Increases the Ability of Normal Plasma to Correct PTA Deficient Plasma.*—Table V lists the partial thromboplastin times obtained when dilutions of silicone and glass-activated normal and normal $BaSO_4$ adsorbed plasma were added to glass-activated PTA deficient substrate plasma from the above-described patients. These data clearly show that glass-activated plasma always shortened the clotting time more effectively than silicone plasma. Thus, in the experiments with substrate plasma from patients J. L. and D. G., a 1:20 dilution of glass-activated plasma exceeded the effect of undiluted silicone plasma. Since the glass-activated PTA deficient substrate plasma contained every clotting factor except PTA in a glass-activated state, the increased effectiveness of normal plasma after exposure to glass must represent a glass-induced increase in PTA activity.

The slight possibility existed that plasma PTA activity might require glass activation when platelet substitutes such as cephalin, but not platelets themselves, were used as the partial thromboplastin. Therefore, the experiments on Patient E. G.'s plasma were repeated to check this point. As the

data in Table VI clearly show, substituting platelets for cephalin in the partial thromboplastin time test did not alter the results. Plasma PTA requires exposure to glass to acquire in vitro activity when either platelets or cephalin serves as the lipid thromboplastic factor.

TABLE V. SHORTENING OF THE PARTIAL THROMBOPLASTIN TIME OF PTA DEFICIENT PLASMA BY SILICONE AND GLASS-ACTIVATED NORMAL AND NORMAL ADSORBED PLASMA

PATIENT	TEST PLASMA DILUTION	PARTIAL THROMBOPLASTIN TIMES* ON ADDING				
		BUFFER (SEC.)	NORMAL PLASMA		NORMAL $BaSO_4$ ADS. PLASMA	
			SILICONE (SEC.)	GLASS-ACT. (SEC.)	SILICONE (SEC.)	GLASS-ACT. (SEC.)
J. L.		130				
	und.		130	55	115	80
	1:5		125	80	100	100
	1:10		120	90	115	100
	1:20		—	95	115	105
D. G.		131				
	und.		109	50	112	70
	1:5		108	64	110	74
	1:10		114	76	113	92
	1:20		115	85	115	98
E. G.		190				
	und.		—	—	145	100
	1:2		—	—	135	120
	1:5		—	—	170	140
	1:10		—	—	165	145

*System: 0.2 ml. of patient's "glass activated" plasma; 0.2 ml. of a dilution of silicone or glass activated normal plasma; 0.2 ml. of cephalin; and 0.2 ml. of calcium chloride. The times listed are averages of two determinations.

TABLE VI. THE DEMONSTRATION OF GLASS ACTIVATION OF PTA WHEN EITHER CEPHALIN OR PLATELETS ARE USED IN THE PARTIAL THROMBOPLASTIN TIME TEST

LIPID THROMBOPLASTIN FACTOR	TEST PLASMA DILUTION	PARTIAL THROMBOPLASTIN TIMES ON ADDING				
		BUFFER (SEC.)	NORMAL PLASMA		NORMAL $BaSO_4$, ADS. PLASMA	
			SILICONE (SEC.)	GLASS-ACT. (SEC.)	SILICONE (SEC.)	GLASS-ACT. (SEC.)
Cephalin	und.	109*	125	60	104	69
	1:5		108	65	102	77
	1:10		100	69	95	81
Platelets	und.	106	122	61	134	79
	1:5		105	68	103	83
	1:10		104	77	99	87

*The shorter times in general on this date are due to the residual effects of a normal plasma transfusion Patient E. G. received several days earlier.

Note that the clotting times often shortened when silicone test plasma was diluted, but always lengthened when glass-activated plasma was diluted. Tocantins[11] has reported dilution-activation of clotting factors. Our data suggest that dilution can activate PTA in silicone plasma and thus compensate for the dilution itself. Glass-activated plasma, already fully activated by the glass, could only diminish in activity on dilution.

DISCUSSION

In 1953, Rosenthal and co-workers[6] described a mild hemorrhagic disease that resembled hemophilia in stemming from defective intrinsic thromboplastin

formation but differed from either hemophilia A or B in being transmitted as an autosomal dominant trait[12] and in being corrected in vitro both by barium sulfate adsorbed plasma and by serum. Rosenthal attributed its cause to a deficiency of a third plasma thromboplastic factor, PTA (plasma thromboplastin antecedent), whose properties he described.[7]

Enough patients have been discovered[13-15] to confirm that PTA deficiency is a distinct hemorrhagic syndrome. However, considerable doubt remains that the PTA syndrome represents a deficiency of a new plasma thromboplastic factor. One hypothesis, advanced because PTA shares properties with both AHG and PTC, is that PTA deficiency may be combined mild AHG and PTC deficiency.[16] To check this possibility, quantitative AHG and PTC levels were measured in our patients. In each, values for both AHG and PTC fell within the normal range.

PTA deficient plasma has been reported to acquire some PTA activity on storage.[7, 10] We found that a stored serum sample from Case 1 corrected the abnormal partial thromboplastin time of his own fresh plasma, and partially corrected that of Cases 2 and 3. Stored serum from Case 3 also corrected the partial thromboplastin time of Case 1. In contrast, stored platelet-poor plasma from Case 1 failed to correct the partial thromboplastin time of his own fresh plasma, or of Cases 2 and 3. We also found, on repeated thromboplastin generation studies of Case 1, that an incubation mixture of cephalin, the patient's fresh adsorbed plasma and the patient's fresh serum always resulted in defective thromboplastin formation. When his stored plasma and serum were used in the incubation mixture, normal values were sometimes obtained. These findings add to the evidence that storage may increase PTA activity and, therefore, to the doubt that the PTA defect is simply an absence of a plasma thromboplastic factor.

Plasma PTA activity, whatever its ultimate explanation, requires contact with glass for its full in vitro expression. Indeed the extent of its glass-induced increase in activity suggests that PTA exists in circulating plasma largely, if not entirely, in an inactive state. Thus, we found that normal silicone plasma did not shorten the prolonged partial thromboplastin time of glass-activated PTA deficient plasma effectively. The same plasma, after being shaken with glass beads, produced marked shortening. These results also emphasize the necessity for standardizing exposure to glass in constructing a quantitative assay for plasma PTA activity.

Barium sulfate adsorbed normal silicone plasma possessed PTA activity, which could also be activated with glass. This confirmed Shafrir and DeVries' observation of a glass-enhanceable clotting activity, not AHG, in barium sulfate adsorbed plasma, and proved correct their assumption that this activity might be PTA. It may also explain Margolis'[17] finding that glass activated aluminum hydroxide adsorbed plasma.

Glass, therefore, can increase at least 3 plasma clotting activities—proconvertin, PTC, and PTA. It is noteworthy that all 3 also increase in activity during clotting, i.e., all 3 are "serum factors" whose activity state is greater

in serum than in plasma. One is tempted to predict that glass will also be found to activate Stuart factor,[18] a clotting activity closely resembling both proconvertin and PTC.

Evidence was presented elsewhere[3] which suggested that the mechanism of proconvertin activation by glass and by brain thromboplastin during clotting was similar. Perhaps the activation of proconvertin, PTC, and PTA by glass and by the intrinsic thromboplastin that forms during spontaneous clotting also involves the same mechanism. Nothing is known of the physicochemical changes in these clotting factors that glass produces. Although glass adsorption of a clotting inhibitor has been postulated,[3] attempts to elute such an inhibitor from glass have failed.[1]

An abstract by Ratnoff and Rosenblum[19] has recently appeared stating that plasma from patients with Hageman trait is deficient in glass-reactive property. These investigators believe, therefore, that glass accelerates clotting by removing an inhibitor from Hageman factor, and that glass-activated Hageman factor initiates in vitro clotting. This hypothesis is difficult to reconcile with the evidence that glass activates PTC, PTA and proconvertin.

However, Ratnoff and Rosenblum also report that mixing duck plasma (which is thought to be deficient in Hageman factor) with human plasma before exposure to glass, prevents the clot-promoting effect of glass. This suggests an alternative hypothesis that could fit both their observation that glass *cannot activate* Hageman factor deficient plasma and our observations that glass *can activate* plasma proconvertin,[3] PTC,[3] and PTA. Perhaps the Hageman defect is not a thromboplastin factor deficiency state but an anomalous plasma physicochemical state that prevents glass contact from activating proconvertin, PTC, and PTA. If adding a small amount of activated PTA or PTC could circumvent this anomaly and allow blood thromboplastin to generate normally in glass vessels, this hypothesis would explain the Hageman defect. Particularly, since the *in vivo* activation of proconvertin, PTC, and PTA obviously cannot involve glass contact, it would explain why patients with Hageman factor deficiency do not bleed abnormally despite their profound clotting defect in glass vessels.

SUMMARY

Plasma from 3 patients with PTA deficiency was shaken with glass beads to make a glass-activated substrate plasma. The prolonged partial thromboplastin time of this PTA-deficient substrate plasma was effectively shortened by glass-activated normal plasma but not by normal plasma that had been exposed only to silicone-coated surfaces. This is strong evidence that exposure to glass increases plasma PTA activity, and suggests that PTA may exist in circulating plasma largely, if not entirely, in an inactive state.

Case 1 was studied through the cooperation of Dr. Madeleine Fallon, and Cases 2 and 3 through the cooperation of Dr. Maurice Yettra. Dr. Joseph Goodman helped with preliminary studies of Case 1.

We are indebted to Dr. Arthur Seaman for the Hageman factor deficient serum.

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DELETION OF PLASMA THROMBOPLASTIN FACTOR D DEFICIENCY

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IN 1954, Spaet and associates[1] reported studies on a patient with a blood coagulation disorder involving thromboplastin formation. The data suggested a clotting defect distinct from any previously described, and it was postulated that the patient lacked a hitherto unrecognized clotting factor. It was suggested that the factor be designated "plasma thromboplastin factor D (PTF-D)," and the associated deficiency "tetartohemophilia." Abnormal coagulation tests included slight prolongation of the whole blood clotting time and moderately impaired prothrombin consumption. In the thromboplastin generation test the patient's serum reagent was markedly abnormal, and his plasma reagent was moderately defective. Plasma thromboplastin factor D was thought to be present in serum, and was apparently partially adsorbed onto barium sulfate. Prothrombin consumption improved when the patient's blood was mixed with blood obtained from patients with hemophilia, plasma thromboplastin component (PTC) deficiency, and plasma thromboplastin antecedent (PTA) deficiency.

Since the original report appeared, no new cases of tetartohemophilia have been reported or established, and several authors have expressed doubt concerning the existence of this entity.[2-4] Accordingly, a cooperative study was undertaken in which the patient traveled to several interested laboratories. An unusual opportunity was thereby provided in which fresh blood of the prototype patient was available to each investigator for study by his individual methods. The data obtained are in agreement, and are compatible with the view that the patient has mild PTC deficiency combined with a circulating anticoagulant of low titer. It was not necessary to explain the clotting defect in terms of a "new" coagulation factor deficiency.

METHODS

Methods used in the present study have been described in detail elsewhere.[5-10] Adaptations applied to specific problems will be presented with the data.

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RESULTS

Table I lists the data obtained by the screening methods used in the participating laboratories. Generally close agreement is shown among them and with those reported in the original study.[1] Some discrepancy was obtained with the prothrombin consumption test, which was most abnormal at the Montefiore and Chapel Hill Laboratories. The thromboplastin generation test was markedly abnormal when the patient's plasma and serum reagents were used. Considerable correction resulted with substitution of normal serum reagent; less improvement followed the introduction of normal plasma reagent. As had been previously reported, assays for platelets, antihemophilic factor, plasma thromboplastin antecedent, the "prothrombin complex," and fibrinogen were within normal limits.

TABLE I. COAGULATION DEFECT BY SCREENING TESTS

TEST	SPAET	RATNOFF	GRAHAM	SCHULMAN	ROSENTHAL
Whole blood coagulation time (minutes)	—	40 (18-31)	12 (5-10)	10.5 (5-10)	14 (5-11)
Plasma recalcified clotting time (seconds)	—	660 (300-600)	—	150 (90-180)	180 (90-270)
Serum prothrombin time (seconds)	27 (>35)			31 (>25)	21 (>25)
(per cent)		<10 (<25)	33 (<10)		
Partial thromboplastin time (seconds)	—	—	160 (70)	—	—
Heparin clotting time (minutes)					46 (20-35)

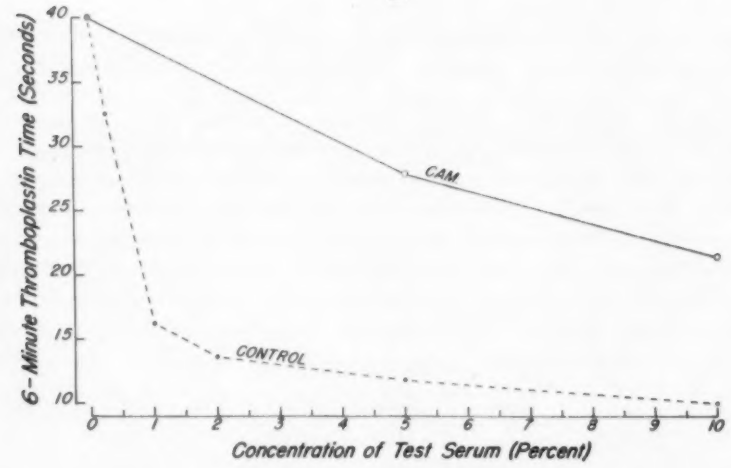
Thromboplastin Generation Test (Clotting Time in Seconds)

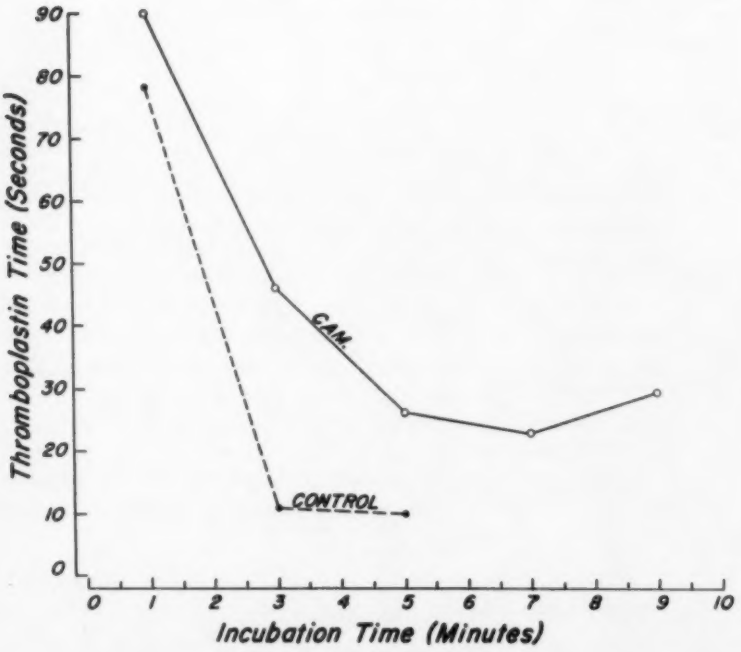
INCUBATION TIME (MINUTES)	1	2	3	4	5	6	7	8
Patient's plasma and serum								
Spaet		55		49		41		
Graham	>20	>20	>20	20	23	22	23	
Schulman			49		13		14	
Rosenthal		82		25		19		19
Patient's plasma, normal serum								
Spaet		37		18		12		
Graham	40	15	15	15	13	15	16	16
Schulman			37		8			
Rosenthal	65	17		9		9		9
Normal plasma, patient's serum								
Spaet		47		34		19		
Graham	19	18	19	20				
Schulman	24		14		18			
Rosenthal		70		18		17		14
Control								
Spaet		25		11		10		
Graham	41	12	12	11	12	11	14	14
Schulman		(8-11 in 3-7 minutes of incubation)						
Rosenthal		13		6		11		12

Normal values given in parentheses

Studies in each laboratory demonstrated independently that there was an abnormally low concentration of PTC in the patient's plasma and serum.

A.





B.

Fig. 1.—Mixtures of the patient's with PTC-deficient serum in the thromboplastin generation test. *A*, Spaet: PTC-deficient serum diluted to 20 per cent was mixed with an equal volume of test serum diluted as indicated, and 6-minute thromboplastin times are recorded. *B*, Schulman: One volume of test serum mixed with 3 volumes of PTC-deficient serum, each diluted to 10 per cent.

Table II and Fig. 1 show that the patient's reagents were markedly less effective than those obtained from normal subjects in correcting the clotting defect of PTC-deficient specimens. On the basis of comparison with variously diluted normal reagents, there appeared to be approximately 10 per cent PTC activity. Conversely, as originally reported,[1] concentrated PTC improved the patient's defect.

TABLE II. IDENTIFICATION OF PTC DEFICIENCY

*Recalcified Clotting Time in Minutes (Ratnoff):**		
PTC-Deficient Plasma Plus an Equal Volume of:		
Patient's plasma	1:20	16
Normal plasma	1:20	10
Normal plasma	1:80	14
Partial Thromboplastin Time in Seconds (Graham):		
PTC-Deficient Plasma Plus an Equal Volume of:		
Patient's plasma		220
Normal plasma		117
AHF-Deficient Plasma Plus an Equal Volume of:		
Patient's plasma		121
Normal plasma		93

Serum Prothrombin Time in Seconds of PTC-Deficient Whole Blood (Spaet):

CONCENTRATION (PER CENT)	PATIENT'S PLASMA	NORMAL PLASMA
2.0	34	46
1.0	30	39
0.5	28	34

PTC-Deficient Whole Blood Coagulation Time in Minutes (Rosenthal):

PTC-DEFICIENT BLOOD PLUS 0.1 ML. OF:	ADDED TO 1 ML.	ADDED TO 2 ML.
Patient's plasma	12	22
Isotonic saline	18	18
PTC-deficient plasma	15	15
PTA-deficient plasma	15	15
Normal plasma	11	11

Serum Prothrombin Time in Seconds of PTC-Deficient Whole Blood (Rosenthal):

PTC-DEFICIENT BLOOD PLUS 0.1 ML. OF:	ADDED TO 1 ML.	ADDED TO 2 ML.
Patient's plasma	20	16
Isotonic saline	13	13
PTC-deficient plasma	16	15
PTA-deficient plasma	24	21
Normal plasma	27	29

*Similar results were obtained with $BaSO_4$ eluates of the patient's and normal plasma.

Additional data suggested the presence of a weak circulating anticoagulant, as shown in Table III. The patient's plasma produced slight prolongation of the partial thromboplastin time in normal plasma, and the patient's prothrombin consumption failed to respond to normal plasma with the degree of improvement seen in uncomplicated PTC deficiency. Anticoagulant activity was insufficient to prolong the recalcified clotting time of an equal volume of normal plasma, whether the mixture was tested with or without prolonged incubation. The presence of such an anticoagulant may explain the patient's poor response to transfused plasma and plasma fractions, and it may account for the slight prolongation of the partial thromboplastin time encountered with mixtures of the patient's plasma with hemophilic plasma (Table II).

Paper electrophoresis of the patient's serum in barbital buffer of 0.05 ionic strength and pH 8.6 showed an abnormal peak in the beta globulin

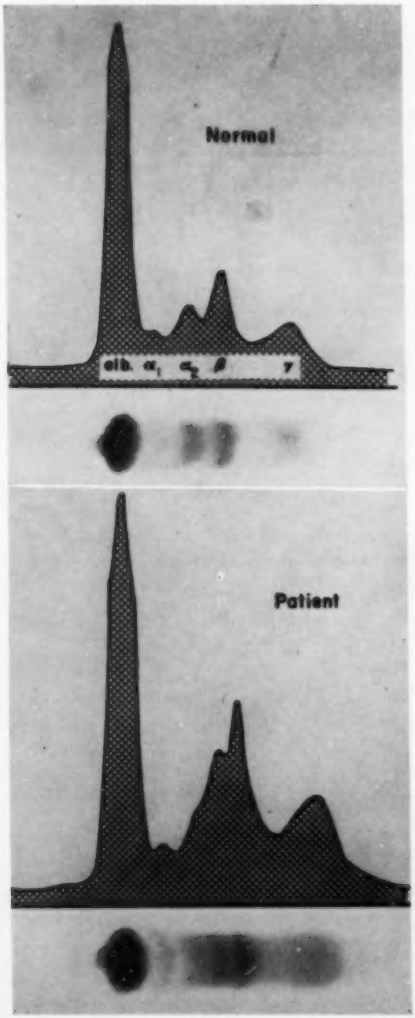


Fig. 2.—Paper electrophoresis of patient's serum.

region (Fig. 2). The significance of this abnormality is not clear, but it differs both from the alpha-X component described in certain hemophiliacs[11] and from the increased gamma globulin associated with the "immune" types of anticoagulants.[12]

TABLE III. EVIDENCE FOR ANTICOAGULANT

Recalcified Clotting Time in Minutes (Ratnoff)	
Equal Volumes of Patient's and Normal Plasma Incubated for 3 Hours.	
Incubated in mixture	6
Incubated separately, then mixed	8
Patient's plasma alone	11
Normal plasma alone	< 10
Partial Thromboplastin Time in Seconds (Graham)	
Equal Volumes of Patient's and Normal Plasma.	
Normal plasma alone	69
Patient's plasma alone	160
Mixture	78

Serum Prothrombin Time in Seconds of Whole Blood (Spaet)
(0.1 ml. of normal plasma reagent added to 1.9 ml. of blood)

CONCENTRATION OF NORMAL PLASMA (%)	PATIENT	UNCOMPLICATED PTC DEFICIENCY
5	42	55
2	25	45
1	24	39
0	23	21

DISCUSSION

The data presented indicate that the patient previously described as PTF-D deficiency (tetartohemophilia) has in fact PTC deficiency, probably complicated by a weak circulating anticoagulant. Removal of one factor from the complicated scheme of blood coagulation is welcome, and the study has illuminated certain other points of interest, not the least of which is the value of cooperative effort. The opportunity to study certain patients with rare or obscure clotting defects, using fresh specimens directly obtained, presents a tremendous advantage over transmission of stored reagents. It is particularly the case with coagulation factors that certain of these may develop increased activity whereas others lose potency even in frozen or lyophilized state.

Our experience with this patient illustrates the fact that mild coagulation disorders present difficulties in diagnosis by techniques in widespread use. In the original study, PTC deficiency was "excluded" because the patient's plasma improved the prothrombin consumption of PTC-deficient blood. This improvement evidently resulted from the small amount of PTC present in the patient's plasma, and the minute amount of PTC required for normal prothrombin consumption is, therefore, again demonstrated. It is evident that mild PTC deficiency may be missed by prothrombin consumption techniques unless low concentrations of test reagents are used. Similar difficulties will be encountered with other clotting disorders until sensitive and reliable assay procedures are available. This problem is underlined by certain patients who give variable and bizarre cross-correction patterns on repeated examination.[13]

Finally, the present study underlines the importance of low-titer anticoagulants. Circulating anticoagulants usually go undetected unless they are of such potency that small concentrations interfere with the clotting of

normal blood or plasma. By this criterion no anticoagulant could be detected in the present patient. Evidence for its presence included the failure of the patient's blood to respond with adequate improvement of clotting following the introduction of normal plasma either in vitro or in vivo and suggested interference of the normal partial thromboplastin time by equal volumes of the patient's plasma, an abnormal plasma reagent curve in the thromboplastin generation test, and a more severe clotting defect than would be anticipated with a PTC level of 10 per cent. "Mild" as these anticoagulants may be, they are probably of considerable clinical significance; they may well account for the development of the resistance to transfusion encountered in certain patients with hemophilia and PTC deficiency and may explain certain obscure acquired clotting disorders.

SUMMARY

A patient previously reported to be deficient in plasma thromboplastin D (PTF-D) has been found to have mild PTC deficiency probably associated with a circulating anticoagulant.

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STUDIES INVOLVING FAT UTILIZATION IN TRAUMATIZED RATS

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DURING a series of studies involving the effects of regimens high in calories and nitrogen on the nitrogen deficit in postoperative patients[1] the question arose as to the utilization of intravenously administered fat in the traumatized individual. It is well known that postoperative patients have a decreased tolerance to glucose but not to intravenously administered fructose[2] during the immediate postoperative period. The abnormality in metabolism of glucose can be demonstrated by the impaired glucose tolerance curve and by the spillage of sugar into the urine. The increased nitrogen excretion by the traumatized or postoperative individual or animal has also been documented.[3-5] The effect of trauma or of surgery on the utilization of fat has not been specifically determined. There are indications derived from minimizing weight loss and nitrogen deficits in postoperative patients which indicate adequate utilization of fat emulsions; however, this is all indirect evidence.[1, 5, 6]

It was the purpose of this study to determine the effect of trauma on the rate of utilization of C^{14}-labeled lauric acid administered intravenously as emulsified trilaurin.

METHODS

Male Sprague-Dawley rats weighing 100 to 125 grams were maintained on standard dog chow and their growth observed. When they reached 150 ± 10 grams, and had normal growth rates, they were placed in individual cages for study. While under ether anesthesia the test animals had the tibia, fibula, and femur of their left leg broken with a heavy hemostat and the metatarsal portion of the same foot crushed. The control animals were not subjected to trauma but were anesthetized for a similar period. Twenty-four hours after the injury occurred, the animals each received intravenously 1.6 ml. (equivalent to 80 mg. of trilaurin) per 100 grams of body weight of the labeled trilaurin emulsion. Between the time of injury and the administration of the fat emulsion the rats received no food but were allowed water.

Immediately following the administration of the fat the rats were placed in respirometer chambers and their expired CO_2 collected in 1 N NaOH for 4 one-half hour, 2 one-hour, and 1 two-hour consecutive periods (total of 6 hours). Aliquots of the alkali-carbonate solutions were then used for the determination of total expired CO_2 by the Van-Slyke manometric technique, and for the total expired $C^{14}O_2$ by radioactivity assays of $BaCO_3$

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samples prepared and counted according to the method described by Solomon and associates.[7] Preparation of the fat emulsion as well as the calculations and correction factors utilized in analyzing the data have been previously described.[8]

RESULTS

The rate of utilization of intravenously administered labeled (C^{14}-00-) trilaurin was studied in 7 control and 8 traumatized rats (Table I). There was no statistically significant difference between the average amount of carbon dioxide expired by the control and traumatized rats during the 6-hour study period. Also, no significant differences were noted between the 2 groups for the total amount of carbon dioxide expired or of fat utilized to CO_2 at any of the

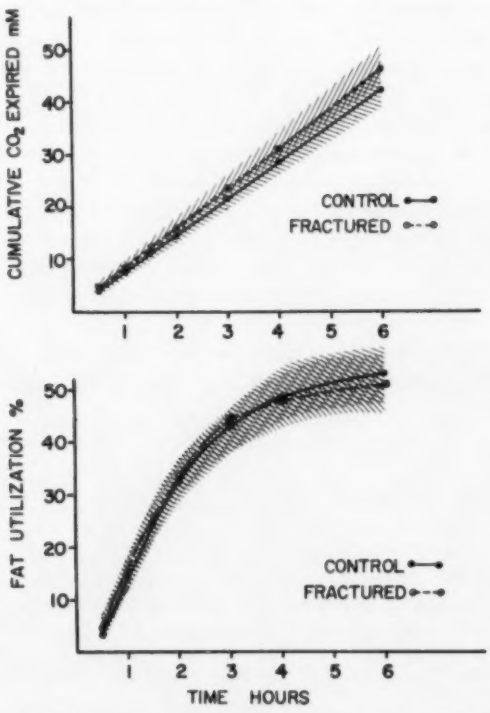

Fig. 1.—Total carbon dioxide expired and fat utilized by the traumatized and control rats, after the administration of a fat emulsion intravenously. The fat was injected at zero time.

7 time intervals studied (Fig. 1). Since the amount of carbon dioxide expired over a definite period of time is an index of metabolic activity, it is probable that the metabolic rates of these 2 groups of animals were not significantly different. The average per cent of the administered C^{14} recovered in the expired CO_2 over the 6-hour period was similar for both groups of rats. The slight increase in CO_2 output and decrease in trilaurin utilization noted in the

traumatized rats as compared to the control rats results in a greater difference in the specific activity of the expired CO_2 between the two groups; however, this difference is not statistically significant ($P<0.1$).

TABLE I. UTILIZATION OF TRILAURIN IN NORMAL AND TRAUMATIZED RATS (6-HOUR CUMULATIVE RESULTS)

CONTROL					TRAUMATIZED				
RAT NO.	WT. (GM.)	CO_2 EXPIRED (mM.)	FAT UTILIZED (%)	SPECIFIC ACTIVITY*	RAT NO.	WT. (GM.)	CO_2 EXPIRED (mM.)	FAT UTILIZED (%)	SPECIFIC ACTIVITY
2	153.2	44.32	59.32	1.338	8	148.8	49.04	49.52	1.010
4	147.2	44.82	47.56	1.061	9	150.1	37.20	48.13	1.294
6	142.0	37.43	52.07	1.391	11	152.7	46.64	56.79	1.218
7	152.5	39.65	50.45	1.272	12	146.5	42.03	51.08	1.215
16	147.0	41.97	49.60	1.182	18	153.0	50.34	55.65	1.105
17	151.0	38.30	52.34	1.366	15	149.5	48.16	60.16	1.249
13	157.0	47.18	59.79	1.250	19	156.8	45.94	43.78	1.103
					20	151.8	45.94	43.78	0.953
Average		42.04	53.02	1.266			45.37	51.65	1.143
		±3.81†	±4.74	±0.117			±4.29	±5.34	±0.120

*Specific activity = per cent of $C^{14}O_2$ per mM. CO_2.

†Standard deviation = $\sqrt{\frac{\Sigma d^2}{n-1}}$.

DISCUSSION

These results show little or no difference in metabolism of intravenously administered fat emulsions between traumatized and control rats. The small differences seen, however, are in the direction we would anticipate from previous results using cortisone-treated rats.[9] Cortisone at a dosage level of 0.66 mg. per 100 Gm. body weight per day causes a significant decrease in both the utilization of exogenous tripalmitin and in the turnover rate of total body fat. At least two factors probably play a role in limiting the differences found in this study: the degree of trauma which influences the resultant stress reaction and the labeled fat used in the study. Unfortunately other parameters for estimating the severity of the stress reaction were not obtained. It should be noted, however, that Cuthbertson and associates[3] have found that the trauma resulting from a fracture of the femur of one leg of the rat was of sufficient magnitude to cause an increase in nitrogen, potassium, sulfur, and phosphorus excretion in the urine. The stress employed in the present work was much greater since there were fractures of the tibia and fibula and crush of the metatarsal area in addition to a fracture of the femur.

Trilaurin was deliberately used in this study since lauric acid constitutes approximately 50 per cent of the component fatty acids of coconut oil which is the fat that is used in some of the emulsions prepared for parenteral use. Previous work with this fatty acid, however, demonstrates that its oxidative utilization proceeds at a more rapid rate than the oxidative utilization of the longer chain palmitic acid and that endocrine changes (i.e., hypophysectomy and cortisone administration) affect its utilization to a lesser degree.[8, 9]

Since the amount of carbon dioxide expired per unit time is an index of the metabolic rate and since the amount of carbon dioxide expired by the two

groups of animals were similar, one might conclude that the metabolic rates of the animals traumatized in this manner were not different from the controls.

SUMMARY

The utilization of C^{14}-00-labeled trilaurin administered intravenously in an emulsified form was studied in control rats and in rats subjected to multiple fractures of the left hind leg. No significant differences were demonstrated in the total amount of CO_2 expired, total amount of C^{14} expired, or in specific activity of the expired CO_2 between these 2 groups. Thus, it may be concluded that intravenously administered fat emulsions are utilized in the traumatized and the untraumatized rat in a similar manner.

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STUDIES ON THE NORMAL SERUM PANAGGLUTININ ACTIVE AGAINST TRYPSINATED HUMAN ERYTHROCYTES

IV. The Presence of Normal Serum Panagglutinin in Patients With Congenital Agammaglobulinemia

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PREVIOUS studies have demonstrated that there exists in normal human serum an agglutinin which reacts against trypsinated erythrocytes of the same blood specimen and against those of other compatible donors.[1, 2] This normal autoagglutinin (NAA) has been found in all sera tested. It differs from pathologic agglutinins in that it produces only immediate agglutination: if incubation of the agglutinated cells is allowed to proceed at room temperature or at 37° C., there is dispersion of the cells and agglutination reversal.

In its ability to agglutinate trypsinated erythrocytes NAA resembles antibody. There is additional resemblance in the finding that NAA appears to be a specific protein which can be adsorbed onto and eluted from trypsinated erythrocytes.[2] Despite these similarities, certain findings tend to weigh against consideration of NAA as an antibody. (1) Normal NAA titers have been found at birth and during the first year of life, when antibody production is minimal.[3] (2) NAA is found in the serum protein fraction I + III of Cohn.[4] This fraction consists primarily of alpha and beta globulins. Antibodies are found characteristically in Cohn fraction II, the γ-globulin fraction. (3) There is no clear evidence that NAA titers are altered during the course of various diseases.

In the present study, sera from patients with congenital agammaglobulinemia were studied for the presence of NAA. It would appear that these patients are incapable of producing antibody[5]; and the finding of NAA in their sera would be additional evidence favoring its nonantibody nature.

METHODS AND MATERIALS

Blood specimens were drawn and NAA titers were determined by techniques previously described.[2, 6] Cells were trypsinated with crystalline trypsin solution (Armour), and each serum was tested against its own erythrocytes.

The patients with congenital agammaglobulinemia were all children who had the typical symptoms of repeated infections. Although all were receiving regular injections of gamma globulin, none of their sera showed more than a trace of gamma globulin by

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paper electrophoresis at the time of study; in most none was detectable. In none of the sera was there any demonstrable alpha or beta isoagglutinin. Several of the patients had been extensively studied at other institutions. Patients Rob. A. and Ron. A. gave no demonstrable yield of gamma globulin with Cohn fractionation of their sera; both maintained a positive Schick test despite repeated attempts at diphtheria immunization. No plasma cells were found in the bone marrows of these children. Immunochemical studies revealed only trace amounts of gamma globulin in the sera of patients J. J. and R. M.

Additional subjects tested were normal volunteers and a patient with severe hypoproteinemia resulting from the nephrotic syndrome.

RESULTS

As seen in Table I, NAA titers of patients with congenital agammaglobulinemia and the patient with hypoproteinemia were within the normal range. When a sufficient amount of gamma globulin concentrate (Lederle) was added to agammaglobulinemic serum to give a final concentration of 1 per cent, no change in NAA titer resulted.

TABLE I. PANAGGLUTININ TITERS OF PATIENTS STUDIED

PATIENT	AGE	DAYS SINCE LAST γ-GLOBULIN INJECTION	TITER
Rob. A.	6	21	1:2
Ron. A.	3	21	1:4
J. J.	9	14	1:8
R. M.	4	5	1:16
M. F.	1	30	1:16
Nephrotic (protein 3.8%)		-	1:32
Controls		-	1:4-1:32

DISCUSSION

Patients with congenital agammaglobulinemia lack the antibodies commonly found in normal subjects; they fail to respond with antibody production when challenged with potent antigens; and they have no demonstrable alpha or beta isoagglutinins for incompatible erythrocytes—agglutinins whose antibody nature is still in doubt.[5, 7] The finding of NAA in these patients provides additional evidence that this agglutinin is not an antibody despite its antibody-like behavior. NAA is thus added to the list of agents whose ability to agglutinate erythrocytes is purely incidental. These included salicic acid,[8] plant agglutinins,[9] basic polyamino acids,[10] multivalent metallic cations,[11] and other unrelated substances. There has been a recent trend to accept many disorders as "immunologic" in nature because they are accompanied by circulating agents that have antibody-like activity. Included are varieties of acquired hemolytic anemia, thrombocytopenic purpura, and leukopenia in which "autoantibodies" have been found.[12] Since nonantibody hemagglutinins are so readily demonstrable, it is probably wise to suspend final judgment as to the pathogenesis of these disorders until it can be established whether or not a specific antigen results in true immunologic formation of the appropriate antibody.

SUMMARY

Patients with congenital agammaglobulinemia had normal titers in their serum of the agglutinin active against trypsinated erythrocytes. The findings provide additional evidence that this agglutinin is not an antibody, and suggest caution in interpreting the immunology of the so-called autoimmune disorders.

We wish to thank Drs. Walter Henley, Herbert Porter, and Stuart Dannoff for their cooperation in providing patients for this study.

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LABORATORY METHODS

THE OX CELL HEMOLYSIN TEST AS A DIAGNOSTIC PROCEDURE IN INFECTIOUS MONONUCLEOSIS

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INFECTIOUS mononucleosis is one of the most common specific illnesses encountered at university health services, military installations, and other medical facilities where the patient population falls largely within the 16- to 25-year-old age range. Reliable methods for the diagnosis of this disease have included the clinical picture with generalized lymph node enlargement, the characteristic changes in white blood cells, and a significant titer of heterophil antibody.

In 1935, Bailey and Raffel[1] first reported the presence of hemolysin for ox red blood cells in the serum of patients with infectious mononucleosis. Several reports[2-4] have subsequently appeared confirming this observation and comparing the ox cell hemolysin test favorably to the heterophil antibody test as a diagnostic procedure. Mason,[2] in 1951, made a study of 60 patients with infectious mononucleosis and 200 controls and concluded that the ox cell hemolysin test was "as sensitive and as specific as other serological tests for infectious mononucleosis." In the following year Leyton[3] reported results of the two procedures with 987 sera concluding that "ox cell haemolysins are a more reliable guide to the presence of glandular fever than sheep cell agglutinins." In 1956, Peterson and associates,[4] in a study of 351 cases which included 23 with "definite" infectious mononucleosis, came to essentially the same conclusion. The present study was undertaken in order to compare the two tests as diagnostic procedures at the University of Michigan Health Service.

MATERIALS AND METHODS

Two hundred and eight serum specimens from 183 patients seen at the University of Michigan Health Service with the presumptive diagnosis of infectious mononucleosis form the basis for this study. Complete medical histories and physical examinations were obtained in every case. Total and differential white blood cell counts were routinely performed. Heterophil agglutination tests including adsorption with guinea pig kidney antigen and ox cell hemolysin tests were performed on each serum specimen.

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When the available information was reviewed 72 patients were considered to have definite infectious mononucleosis, 19 to possibly have infectious mononucleosis, and 92 to have some disease definitely not infectious mononucleosis. Criteria for diagnosis included: (1) a typical clinical picture with generalized lymph node enlargement, fever, and sore throat; (2) lymphocytosis, usually greater than 50 per cent, with over 10 per cent atypical lymphocytes of the Downey type; and (3) a significant heterophil antibody titer (1:448 or greater). The clinical information regarding the 72 patients with definite infectious mononucleosis is summarized in Table I.

TABLE I. CLINICAL AND LABORATORY FEATURES OF 72 PATIENTS WITH DEFINITE INFECTIOUS MONONUCLEOSIS

FEATURE	PATIENTS	PER CENT
Lymphadenopathy	65	90
Laryngitis	59	82
Fever	56	78
Splenomegaly	19	26
Hepatomegaly	16	22
Leukocyte count:		
under 5,000 per c.mm.	3	4
5,000 to 10,000 per c.mm.	13	18
over 10,000 per c.mm.	55	76
Lymphocytosis:		
under 40 per cent	1	1
40 to 60 per cent	22	31
over 60 per cent	48	68
Atypical lymphocytes (per cent of lymphocytes examined)		
less than 50 per cent	14	20
50 per cent or more	57	79

Heterophil Antibody Test.—Serum was routinely adsorbed with guinea pig kidney antigen as described by Davidsohn.[5] Serum was inactivated by heating at 56° C. for 30 minutes. Two-tenths milliliter of inactivated serum was then placed in a test tube with 1.0 ml. of well-mixed guinea pig antigen suspension, mixed, and incubated ta 37° C. for 30 minutes, or at room temperature for one hour. The tubes were then centrifuged at 1,500 r.p.m. for 10 minutes and serial dilutions prepared. To each of 10 tubes, except the first, was added 0.25 ml. of 0.85 per cent sodium chloride solution. Two and five-tenths milliliters of the supernate was then added to the first and the second tubes and serial dilutions made through the tenth tube, from which 0.25 ml. was discarded. One-tenth milliliter of a 2 per cent suspension of washed sheep red blood cells was then added to each tube and mixed well. Tubes were then incubated at room temperature for 2 hours and read with low-power objective of the microscope where clumping was not grossly visible.

Ox Cell Hemolysin Test.—Serum was inactivated by heating at 56° C. for 30 minutes. To each of 10 test tubes was added 0.5 ml. of 0.85 per cent sodium chloride containing 0.1 Gm. magnesium sulfate per liter. Five-tenths milliliter of inactivated serum was then added to the first tube and serial dilutions made. Five-tenths milliliter of complement solution* was then added to each tube, followed by 0.5 ml. of a 2 per cent suspension of beef red blood cells.† Complement is restored by adding 2.25 ml. stabilizing solution to the 3 ml. size ampule. A 1:15 dilution of restored complement is used for the test. After thorough mixing, tubes were placed in a water bath at 37° C. for 15 minutes. The tubes were then centrifuged at 2,000 r.p.m. for 2 minutes and read in comparison with a 50 per cent hemolysin standard. The standard was prepared by adding 0.5 ml. of complement solution

*Merck Sharp & Dohme, Inc. (Division of Merck & Company, Inc.), Philadelphia, Pa.

†Obtained as 10 per cent suspension in Alsever's solution at 2-week intervals from Mayer & Miles Laboratories. Beef cells have been found to behave in a fashion identical to the less readily available ox red blood cells in the hemolysin procedure.

TABLE II. COMPARISON OF HETEROPHIL AGGLUTINATION AND OX CELL HEMOLYSIN TESTS IN 72 PATIENTS WITH DEFINITE INFECTIOUS MONONUCLEOSIS

DURATION OF ILLNESS	HETEROPHIL AGGLUTINATION TEST				OX CELL HEMOLYSIN TEST			
	NUMBER OF TESTS	"NEGATIVE" 1:56 OR LESS (PER CENT)	"SUSPICIOUS" 1:112 TO 1:224 (PER CENT)	"POSITIVE" 1:448 OR GREATER (PER CENT)	NUMBER OF TESTS	"NEGATIVE" 1:48 OR LESS (PER CENT)	"SUSPICIOUS" 1:96 TO 1:384 (PER CENT)	"POSITIVE" 1:768 AND GREATER (PER CENT)
First week	33	27	27	46	28	11	4	85
Second week	22	27	9	64	21	5	10	85
Third week	6	--	16.7	83.3	6	16.7	--	83.3
Fourth week	6	50	16.7	33.3	6	--	33.3	66.7
Fifth week and later	10	60	10	30	10	20	40	40
Entire group	84	30	18	52	78	10	14	76

and 0.5 ml. of 2 per cent suspension of beef red blood cells to 2 ml. of distilled water. The end point titer was the dilution in the tube reading closest to the standard. No difficulty was experienced with beef red blood cells up to 2 weeks of age.

RESULTS

In this laboratory a heterophil agglutination titer of 1:56 or less has been considered "negative," 1:112 to 1:224 "suspicious," and 1:448 or greater "positive." For comparative purposes an ox cell hemolysin titer of 1:48 or less has been considered "negative," 1:96 to 1:384 "suspicious," and 1:796 or greater "positive." Results of the two procedures have been carefully evaluated in the 72 patients with definite infectious mononucleosis (Table II) and the 92 patients without infectious mononucleosis (Table III). Results have not been tabulated in the 19 patients with questionable infectious mononucleosis since it was felt that interpretation would not be meaningful.

TABLE III. COMPARISON OF HETEROPHIL AGGLUTINATION AND OX CELL HEMOLYSIN TESTS IN 92 PATIENTS WITHOUT INFECTIOUS MONONUCLEOSIS

METHOD	"NEGATIVE"	"SUSPICIOUS"	"POSITIVE"
Heterophil agglutination	1:56 or less 100%	1:112 to 1:224 None	1:448 or greater None
Ox cell hemolysin	1:48 or less 95%	1: 96 to 1:384 5%	1:796 or greater None

In the group with definite infectious mononucleosis the over-all incidence of "positive" results with the ox cell hemolysin test was greater (75 per cent) than with the heterophil agglutination test (52 per cent). It also appeared that the hemolysin test was more frequently "positive" the first and second weeks of illness than the heterophil agglutination test. In general, the hemolysin procedure was "positive" in greater dilution than the heterophil agglutination test; the end point titer was higher with the hemolysin test in 60 instances, approximately equal in 12, and significantly less in only 4. In no case was the heterophil agglutination positive in the face of a negative ox cell hemolysin test. Inadequate information is available to permit accurate comparison of the duration of significant titer with the two procedures but it is our impression that the hemolysin titer persists longer than the heterophil.

Technically the ox cell hemolysin test has proved to be simpler, faster, and less expensive to perform than the heterophil agglutination test. No adsorptions are necessary and the test can be carried to completion in approximately 30 minutes following inactivation.

SUMMARY AND CONCLUSIONS

Two hundred and eight serum specimens from 72 patients with definite infectious mononucleosis, 19 with questionable infectious mononucleosis, and 92 individuals without infectious mononucleosis have been tested with the heterophil agglutination and ox cell hemolysin procedures.

The ox cell hemolysin test has been found to compare favorably with the heterophil agglutination test as a diagnostic procedure. In the group with

definite infectious mononucleosis it was more frequently positive in the first and second weeks of illness and gave a higher proportion of significant results on random specimens obtained at various stages of the disease.

Technically, the ox cell hemolysin test has been found to be simpler, faster, and less expensive to perform than the heterophil agglutination test with guinea pig kidney adsorption. It is recommended as a routine laboratory procedure in institutions caring for large numbers of young people. It is suggested that greatest diagnostic assistance will be obtained by performing both tests rather than either procedure alone.

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CONCENTRATION OF VITAMIN B_{12} FROM URINE BY ADSORPTION ON CARBON

A Sensitive Assay of Radiocyanocobalamin in the Schilling Test for Pernicious Anemia

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THE Schilling test[1] has been widely accepted as a rapid, dependable laboratory method for demonstrating intrinsic factor deficiency. Usually the patient is given an oral dose of 0.5 μg vitamin B_{12} labeled with 0.5 μc of cobalt^{60}. Two hours later, 1,000 μg stable vitamin B_{12} is injected intramuscularly so as to flush a large portion of the absorbed radioactive vitamin into the urine. If there is no abnormality of vitamin B_{12} absorption or kidney function, at least 10 per cent of the dose appears in the urine during the first 24 hours. A 24-hour urinary excretion of 0 to 3 per cent of the dose indicates deficient absorption.[2] A second oral dose of radioactive vitamin B_{12}, administered together with an active intrinsic factor preparation, will show whether or not intrinsic factor relieves the absorption deficiency.

Faulty urine collection and complications such as renal insufficiency and abnormalities of the digestive tract often necessitate performance of multiple Schilling tests. Despite the flushing procedure the normal liver retains approximately one-half of the dose with a biologic half-life of about 1 year.[3, 4] The liver is thus subjected to radiation totaling 2.5 rem per 0.5 μc radiocyanocobalamin administered.[5] Therefore, it is desirable to minimize the dose of radioactive material administered to patients without impairing the sensitivity of the test. Kinnory[6] has described a method of concentrating urinary vitamin B_{12} into a very small volume. The sensitivity of the radioassay is thereby increased almost a hundred fold and a tenfold reduction of the dose can be made readily. Kinnory adds ammonium chloride, cobaltous chloride, and ammonium sulfide to the urine and collects the radioactive vitamin B_{12} on the cobalt sulfide precipitate. Since this method is cumbersome and involves exposure to noxious H_2S fumes, we investigated the suitability of other adsorbing agents. Diatomaceous earth and activated carbon have been used previously in the purification of vitamin B_{12} (e.g., ref. 7). Miller[8] has used activated charcoal in concentrations of 2.5 to 10 per cent to remove free vitamin B_{12} from serum.

METHODS

We tested the following adsorbents under various conditions of acidity and salt concentration: Dowex 50-X2 cation exchanger; cellulose powder (Whatman, standard grade,

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ashless); diatomaceous earth (Filter Cel); and activated carbon (Norit, F.Q.P.). We also tested a suspension of nonradioactive, freshly precipitated cobalt sulfide which had been washed with dilute cobaltous chloride and water to remove excess sulfide ion.

After evaluation of the results we adopted the following method for routine analysis of 24-hour urine specimens: We acidify any alkaline urines with sulfuric acid and then add 1 ml. 18 N sulfuric acid and 0.4 Gm. Norit to a 70 ml. aliquot of the urine in a 25 by 200 mm. screw-cap test tube. We leave the tube for 15 minutes in a modified Kahn shaker* and then centrifuge and decant the supernatant. In order to transfer the sediment to a 16 mm. test tube, we slurry it with about 2 ml. supernatant and pick it up by means of a 5 ml. serologic pipette and a pipette control (consisting of a 5 ml. Yale B-D Lok syringe and a 1-inch piece of heavy-walled rubber tubing, 3/16 in. inside diameter and 3/16 in. wall). From each urine specimen we generally take four 70 ml. aliquots. The combined packed sediment from 4 times 0.4 Gm. Norit occupies a volume of 4 to 4.5 ml. We assay the sediment in a well-type scintillation counter and compare it with an aliquot of the original radiocyanocobalamin solution. Our counter has an efficiency of 0.83 million c.p.m. per microcurie of cobalt^{60} for sample volumes up to 1.5 ml. and 0.73 million c.p.m. per microcurie for a 4.5 ml. sample. The background counting rate is 170 c.p.m.

If the radioactivity of the sediment indicates a lower than normal urinary excretion value, we check the efficiency of adsorption by treating the supernatant with another 0.2 Gm. Norit and determine the activity of the resulting sediment. In this case we also analyze the second 24-hour urine specimen which we collect routinely after giving the patient a second intramuscular injection of 1,000 μg vitamin B_{12} 24 hours after the oral dose of radiocyanocobalamin.

TABLE I. RECOVERY OF RADIOCYANOCOBALAMIN ON VARIOUS ADSORBENTS (0.14 PER CENT SUSPENSIONS IN URINE)

SOLUTES ADDED TO URINE	PER CENT RECOVERY OF Co^{60}–B_{12}			
	DOWEX 50-X2	CELLULOSE	FILTER CEL	NORIT
None	3	0.6	2	29
Saturated NH_4Cl	2	1	3	50
Saturated $(NH_4)_2SO_4$	81	55	68	86
Saturated $(NH_4)_2SO_4$ + 0.25 N H_2SO_4	72	79	94	97
0.25 N H_2SO_4	28	1	5	60

RESULTS

Table I compares the B_{12} adsorbing power of 4 adsorbents and shows the effect of different solutes. The use of saturated ammonium sulfate requires a high solute concentration and entails a 40 per cent increase in sample volume. Equally good adsorption is achieved more conveniently by using higher concentrations of Norit. Two grams of Norit or more per 70 ml. solution and 0.13 to 0.25 N sulfuric acid gave vitamin B_{12} recoveries of 82 to 100 per cent. At low Norit concentration the B_{12} recovery is small and variable. Vitamin B_{12} adsorption approached equilibrium after about 10 minutes of mechanical shaking or sooner, depending on Norit concentration.

We observed that Norit adsorbs some of the colored constituents of urine in preference to vitamin B_{12}. After the urine has been cleared of this interfering material, a fresh portion of Norit picks up the remaining vitamin with great avidity. The degree of decolorization provides an immediate, approximate clue to the efficiency with which vitamin B_{12} is adsorbed from a given

*A hemacytometer pipette platform (Fisher Scientific, No. 6-213B) was sawed into two parts and extended to accommodate the large screw-cap tubes. The platform was then screwed onto the platform of a Fisher-Kahn shaker (Fisher Scientific, No. 14-255).

urine specimen. Since some urines have a high concentration of the interfering material, we have been using 0.4 Gm. Norit per 70 ml. urine in order to obtain consistently high adsorption of radioactive vitamin B_{12}.

We tried Kinnory's method[6] of isolating urinary radiocyanocobalamin on cobalt sulfide precipitate and found that the total recovery after two successive precipitations ranged from 85 to 96 per cent in 5 assays. Single precipitations gave widely varying recovery ranging from 43 to 88 per cent. In contrast to the statement of Kinnory and associates, it appeared that the radioactivity from the labeled B_{12} enters the precipitate by adsorption rather than by formation of $Co^{60}S$. This view is supported by the elutability of most of the radioactivity in the precipitate with 50 per cent acetone. In addition we observed that the recovery of Co^{60}–B_{12} on sulfide precipitate from 2×10^{-4} M cobalt chloride in a saturated ammonium chloride solution dropped from 102 to 65 per cent when the concentration of carrier B_{12} was increased from 4×10^{-10} M to 4×10^{-7} M.

Washed cobalt sulfide (0.014 mole per liter or more) was a very effective adsorbent in the presence of 0.26 N sulfuric acid. Ninety per cent of the radiocyanocobalamin in 450 to 550 ml. urine was removed in a single step by an amount of cobalt sulfide which measured only 5 ml. after centrifugation.

DISCUSSION

The Norit adsorption method described above achieves a sixty fold concentration of urinary radiocyanocobalamin and makes it possible to carry out Schilling tests with 0.05 μc of radioactive vitamin B_{12}. This dose is one-tenth of that customarily employed. The volume of urine which can be analyzed by this method is limited by the capacity of the well-type scintillation counter and by the concentration of Norit required to achieve consistently high recoveries of radiocyanocobalamin. Frelick and his associate have indicated that "Darco G-60 Special" activated carbon adsorbs vitamin B_{12} more effectively than does an equal weight of Norit (W. A. Helbig, private communication). In our hands the efficiency gain was only about 12 per cent based on equal volumes of packed carbon sediments.

Ellenbogen[9] has described a method of analyzing 1,000 ml. urine directly in a modified Marinelli beaker placed over a scintillation crystal. This method involves a minimum of work and its sensitivity approaches that obtained by the Norit adsorption method. However, the necessary counting equipment is not available in many laboratories.

Kinnory's cobalt sulfide adsorption method is about 3 times as sensitive as the Norit method and separates radioactive vitamin B_{12} quite efficiently from I^{131} contamination. However, it exposes the operator to H_2S fumes and involves the additional work of saturating the urine with ammonium chloride. Exposure to H_2S fumes can be minimized by preparing nonradioactive cobalt sulfide suspension in an amount sufficient for several urine assays. However, the adsorptive capacity of cobalt sulfide suspension decreases considerably during an

interval of several days. Furthermore, aliquots of cobalt sulfide suspension are difficult to measure because they settle rapidly and form an opaque film on the wall of the pipette.

The investigations of Baker and associates[10] appear to indicate that a patient whose level of intrinsic factor is so low as to permit the absorption of only a small fraction of an oral dose of 0.5 μg B_{12} may well be able to absorb a large fraction of a 0.05 μg dose. For this reason we add sufficient stable vitamin B_{12} to our stock solution to keep the oral dose at the 0.5 μg level. For similar reasons Williams and co-workers[11] recommend a dose of 2.0 μg B_{12} for intrinsic factor assays.

SUMMARY

In order to develop a sensitive urinary radiocyanocobalamin assay and thus to permit a tenfold reduction of the customary radiocyanocobalamin dose, several adsorbing materials were tested under various conditions as to their suitability for concentrating the vitamin B_{12}. The most satisfactory procedure employed activated carbon in acidified urine and achieved a sixty fold concentration of the radiocyanocobalamin. It was preferable to the use of cobalt sulfide whose high adsorptive capacity is offset by the exposure of the technician to noxious hydrogen sulfide fumes.

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A SIMPLE DEVICE FOR MAKING EMULSIFIED VACCINES*

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EMULSIFIED vaccines, first described by Freund and McDermott,[1] have proved to be an effective method of inducing high antibody responses in laboratory animals.

A device has been developed for making water-in-oil emulsions in small quantities. Two Luer-Lok syringes of similar size are coupled together forming an opposing piston machine in which emulsions are made. This method of emulsification avoids drawbacks frequently encountered in preparing emulsions: i.e., entrapment of air, waste of materials, and difficulty in filling syringes.

The double-hubbed coupler is made by wedging a 20 gauge hypodermic needle into an 18 gauge needle after cutting off the bevel of both needles. Each needle is cut approximately ¼ inch from the hub with the sharp edge of an abrasive wheel mounted on a hand tool. The cylindrical shape of the needle is maintained by inserting a cleaning stylus before cutting. After wedging, the needles may be bonded with silver solder. Care must be taken during union of the needles to avoid splitting the larger shank for, if this occurs, the unit will leak when pressure is applied.

Syringes and coupler may be mounted in a wooden holder to avoid lateral motion of the needle and minimize needle breakage (Fig. 1).

Emulsions are made by forcing components through the small orifice in the coupler using positive pressure on syringe plungers. Initially, the desired volumes of oil and water are loaded into separate syringes. By careful filling of syringes and coupler, entrapment of air can be avoided. Water-in-oil emulsions are more easily started if the syringe containing the oily phase is attached to the number 20 hub.

Emulsification is started by injecting approximately one-tenth the volume of aqueous material into the syringe containing oil by repeated, forceful, incomplete strokes of the plunger. Dispersion of water droplets in oil and oil droplets in water occurs with sufficient positive pressure. Between each stroke the contents of the syringe initially containing the oily phase are briefly permitted to separate until the aqueous portion can be returned to the original syringe by slight pressure on the opposite plunger. Formation of a water-in-oil emulsion is indicated by marked increase of resistance to movement by the plungers due to increased viscosity of the mixture.

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Emulsification is completed by repeated forceful cycling of the total contents between the two syringes. Complete emulsification is indicated when the mixture appears homogeneous and offers uniform resistance as the mixture is cycled between syringes. Continued cycling increases emulsion viscosity.

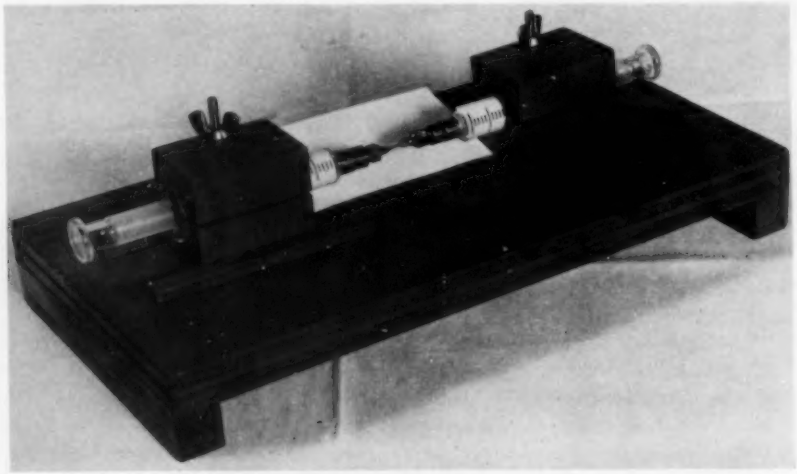

Fig. 1.—Syringes mounted in holder. Double-hubbed coupler unites Luer-Lok syringes containing water-in-oil emulsion. Syringe on left is mounted in wooden block which can be adjusted to accommodate coupling needles or syringes of differing length. Syringes are clamped rigidly in each block by tightening winged-nuts.

The number of strokes required to start emulsification has varied with different mixtures, components, and couplers. Satisfactory influenza vaccine in mineral oil emulsions are usually formed after 6 to 8 strokes.

Since emulsions may be transferred with positive pressure between syringes, the double-hubbed coupler provides means of filling syringes for inoculation. Complete filling of a smaller syringe is obtained by slightly overfilling the smaller syringe to expel air bubbles which are usually around the plunger. By this method, syringes can be filled with highly viscous emulsions without entrapment of air usually resulting when other means of syringe loading are used.

SUMMARY

A simple method of emulsifying vaccines in oil is described. The emulsifying device also enables convenient handling of emulsified vaccines.

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A MODIFICATION OF MALKIN'S SCREENING TEST FOR THE DETECTION OF ARTIFACTUALLY HIGH ORGANIC IODINE COMPOUNDS IN SERA

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IN MALKIN'S article[1] is outlined a simple semiquantitative procedure for detecting large amounts of iodine-containing compounds, used in roentgenographic procedures, and similar substances. Malkin's article was published after Samson and associates[2] had described a "Cross-Contaminator Exclusion Test." These tests were developed for use with the protein-bound iodine method, in which samples of artifactually high value cause contamination of apparatus and other samples but may not indicate contamination which is less than 0.8 mg. of iodine per 100 ml.

With contaminated sera containing less than 0.8 mg. per 100 ml., determinations of PBI (protein-bound iodine) or BEI (butanol extractable iodine) have no clinical significance, and contamination of other samples does occur. According to the original procedure discussed by Malkin, 3 drops of serum are digested with 2 drops of 8 N potassium hydroxide at 100° C. for 30 minutes. Malkin stated that the digestion partially hydrolyzes the serum proteins with the breaking of peptide bonds which hold the artifactual iodine. The standard diminution in color when ceric sulfate is reduced to cerous form by arsenite in the presence of iodide is used. (The yellow ceric ion is reduced to colorless cerous ion.) Five minutes after the addition of ceric sulfate and arsenite, the samples are read against a white background. Marked fading indicates contamination. Malkin recommends that a sample of known high value be used as a control.

The following changes were made to improve the sensitivity of Malkin's procedure. Dilution with distilled water is omitted but 0.50 ml. of potassium iodide (1.4 mg. per 100 ml.) is added before the addition of the arsenite and ceric sulfate. The additional iodide increases the sensitivity of the test so that contamination above 0.1 mg. per 100 ml. of serum should be detected. The addition of more potassium iodide would not facilitate differentiation of unusually high pathologic sera from artifactually contaminated samples since the difference in color change would not be apparent. Two drops of 27 N sulfuric acid are added after the digestion with potassium hydroxide so that the arsenite and ceric sulfate solutions, which are used and available for the BEI technique, may be employed routinely without preparation of special reagents. The reagents for the BEI technique were described in an earlier article.[3, 4]

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MATERIALS AND METHODS

Reagents.—

1. Potassium hydroxide (8 N). Dissolve 527 Gm. of KOH pellets in H_2O and dilute to one liter.
2. Sulfuric acid (27 N).
3. Ceric sulfate (0.1 N) and arsenious acid solution (0.3 N in 1.0 N H_2SO_4) prepared as for the butanol extractable iodine method.[3, 4]
4. Potassium iodide, 1.4 mg. of iodide per 100 ml. (17.5 ml. of secondary potassium iodide solution is diluted to 500 ml.[3]).

Methods.—Two drops of 8 N KOH are added to 3 drops of each sample of serum. The test tubes are heated at 100° C. for 30 minutes in a Chaney-Johnson seroscope, Model RH 200 (or tubes may be placed in a Kahn rack which is placed in a boiling water bath, as suggested in the Malkin article[1]). After 30 minutes tubes are removed and 1 drop of 27 N H_2SO_4, 0.50 ml. of KI, 1.0 ml. of 0.3 N arsenious acid, and finally 1.0 ml. of 0.1 N $CeSO_4$ solutions are added.

Shake the solutions at room temperature and, after 5 and 15 minutes, compare the fading with the known contaminated standard against a white background. Fading has been judged adequately by inexperienced technicians after a few consecutive days of routine tests on the daily influx of new sera. Centrifugation to separate the protein precipitate has not facilitated recognition of fading of the clear supernatant fluid. When a sample appears pale after 5 minutes, the second examination after 10 minutes increases accuracy of the test because contaminated samples lose more color progressively while noncontaminated sera, which originally exhibited little pigment coloration, do not fade continuously during the 10-minute interval. In our experience it has not seemed necessary to compare the color of a suspicious sample with the color of the same serum which has not been digested with alkali but has had added the usual quantity of sulfuric acid, potassium iodide, arsenious acid, and ceric sulfate. Until familiarity with the test develops, such a control for a suspicious serum should eliminate the effects of endogenous coloring of that sample of serum.

SUMMARY

This test is sensitive, except in highly colored sera, for recognition of contaminations encountered to date amounting to 75 gamma of iodine per 100 ml. of serum. Fading of ceric sulfate is often apparent when a patient has received inorganic iodine, but knowledge of such treatment prevents suspicion of organic iodine contaminants. Lugol's solution, in quantities used in this Department for treatment of thyroid dysfunction, has resulted in concentrations of inorganic iodine between 100 and 200 gamma per 100 ml. of serum. Occasionally a positive test occurs in a serum which subsequently is found to have a normal BEI content. Nevertheless, routine use of this test for over a year has prevented some of the difficulties created by unknown contaminating iodine substances.

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A METHOD FOR ACCURATE DETERMINATION OF PLASMA ALBUMIN

Comparison of Albumin Values Obtained With Three Other Methods

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THE determination of plasma albumin is a common procedure in clinical laboratories. Relatively few laboratories possess more elaborate means for quantitation of plasma protein components, such as electrophoresis. The majority rely on salting-out procedures primarily because they provide more expedient methods for a larger number of determinations.

A number of papers have appeared proposing various means of salting-out the globulins for albumin analysis.[1-9] Some reports included comparisons with electrophoretic data.[6, 8, 9, 11, 15] It would appear that the question of albumin analysis had been successfully resolved; however, in a great many clinical laboratories today, this analysis provides the least reliable data because the procedures for albumin and globulin separation are not specific with regard to the extent and nature of shaking movements.

The purpose of this paper is to present details of a modification of the salting-out methods of Kingsley[4] and Milne[9] and to determine to what extent variation in shaking influences the results of plasma protein fractionation. The values obtained with this method, together with two other methods,[1, 8] are compared with the values obtained with the Tiselius electrophoresis apparatus.[10]

MATERIALS AND METHODS

Subjects used in the comparative study of plasma albumin were hospital patients with various diseases and disorders, including patients with marked hypoalbuminemia and hyperglobulinemia.

The procedure used for globulin precipitation is described in this paper. Two other salting-out methods for protein precipitation, with which this method is compared, have also been used. The salts and the concentrations were as called for by those methods.

The total protein and the albumins were determined by the standard micro-Kjeldahl procedure[16] and corrected for the nonprotein nitrogen. The nitrogen to protein conversion factor used was 6.25. The electrophoretic separation of plasma albumin, along with other protein components, was carried out with the Tiselius electrophoretic apparatus,[10] after a 20-hour dialysis against the veronal buffer of ionic strength 0.1 and pH 8.6.

Procedure.—Place 0.25 ml. of plasma into a 16 by 125 mm. test tube. Add 7.75 ml. of 26.8 per cent Na_2SO_4 solution kept at 37° C. and overlay with 3 ml. of diethyl ether. Stopper and mix gently by inversion—11 times in 30 seconds (gentle inversion is defined as inverting the tube upside down and then to its original position for one inversion).

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Centrifuge for 10 minutes at 900 g. Tilt the tube to a 45 degree angle. This will loosen the packed globulin layer away from the sides of the test tube. Cover the end of a 2 ml. pipette with the index finger then carefully insert its tip along the side of the test tube, without touching the globulin layer, into the albumin solution below and withdraw a 2.0 ml. aliquot for analysis.

The albumin solution obtained by this procedure was then analyzed for nitrogen according to the micro-Kjeldahl method.[16]

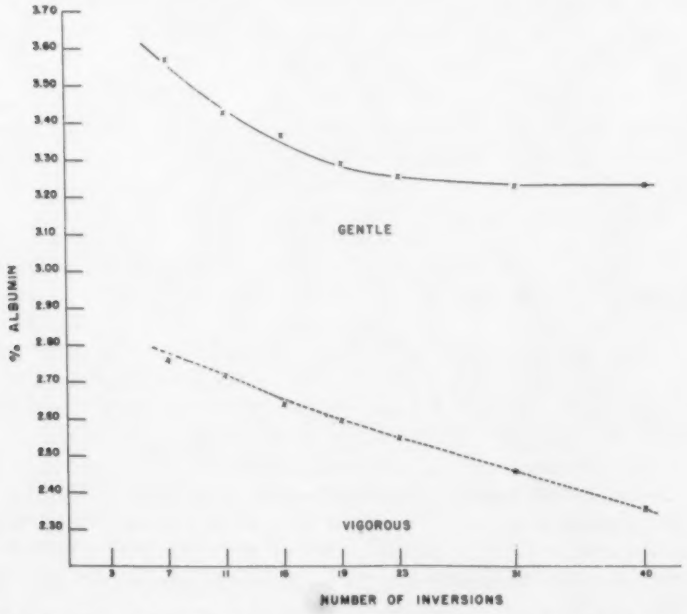

Fig. 1.—The effect of gentle inversion and vigorous shaking upon the precipitation of globulins from human plasma.

RESULTS

A plasma pool obtained from 35 hospitalized patients was used to compare albumin values obtained by gentle inversion with values obtained by vigorous shaking. The globulins were precipitated with 26.8 per cent sodium sulfate. The frequencies of inversion or vigorous shaking were 7, 11, 15, 19, 23, 31, and 40. Fig. 1 shows the effect of gentle inversion and vigorous shaking upon the precipitation of globulins from human plasma.

DISCUSSION

The albumin values of the 22 per cent sodium sulfate method[1] are higher than those obtained with electrophoresis (Table I, Fig. 2). This has been brought out by other investigators[12-14] who have shown also that the albumin filtrates obtained with 22 per cent sodium sulfate contain the alpha and beta globulins which are calculated as albumin.[14]

TABLE I. COMPARISON OF PLASMA ALBUMIN LEVELS OBTAINED WITH 22 PER CENT AND 26.8 PER CENT Na_2SO_4 WITH THE VALUES OBTAINED BY ELECTROPHORESIS

SUBJECTS	TOTAL PROTEIN (GM. PER CENT)	ALBUMIN (GM. PER CENT)		
		SODIUM SULFATE 37° C.		ELECTROPHORESIS
		22.0 PER CENT	26.8 PER CENT	
1	7.99	5.25	3.42	3.17
2	6.14	2.63	2.41	2.06
3	6.02	3.18	2.20	2.06
4	6.92	3.74	2.94	2.63
5	6.30	4.02	3.60	2.89
6	7.14	3.80	3.80	3.07
7	6.06	2.47	2.23	1.81
8	7.09	3.15	3.04	2.86
9	7.03	3.83	3.46	3.49
10	6.23	3.41	2.90	2.08
11	6.27	3.76	2.85	2.70
12	5.78	3.39	2.74	2.72
13	5.87	3.18	2.66	2.30
14	6.74	4.36	3.80	3.21
15	6.27	3.87	3.36	2.82
16	9.01	4.24	2.85	2.83
17	7.04	4.34	3.76	3.98
18	8.78	4.54	3.62	3.86
19	8.03	5.41	4.43	4.29
20	8.50	5.96	3.86	3.87
21	8.12	4.52	3.25	3.25
Average	7.01	3.95	3.19	2.95
S.D.		0.77	0.57	0.66

Plasma from 21 hospital patients was used for simultaneous analysis using 22.0 per cent Na_2SO_4[3] and the proposed method using 26.8 per cent Na_2SO_4 for precipitating the globulins.

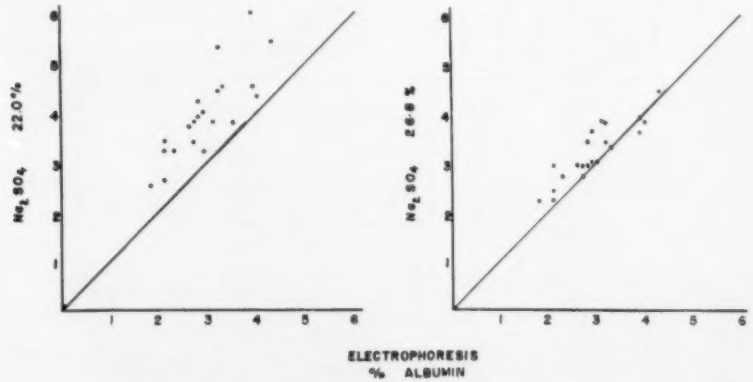

Fig. 2.—Scattergrams illustrate a comparison of plasma albumin values in grams per cent obtained with 26.8 per cent Na_2SO_4 and 22 per cent Na_2SO_4 precipitation methods with respect to electrophoresis values. The solid black line represents 100 per cent correlation for the methods indicated.

Jager and his associates,[14] in their evaluation of 6 methods for estimation of serum albumin, noted that shaking of the salt protein mixture lowers the albumin value. They proposed shaking of the salt protein mixtures immediately after mixing and prior to filtration. Employing this technique, they have found that the $MgSO_4$ method[8] yielded results which were a little closer to the electrophoresis values than those of the 26.8 per cent Na_2SO_4 method.[9]

The results presented in this paper (Table II, Fig. 3) show that although the albumin values of $MgSO_4$ and Na_2SO_4 methods agree well with electrophoresis, a closer agreement exists with the Na_2SO_4 values.

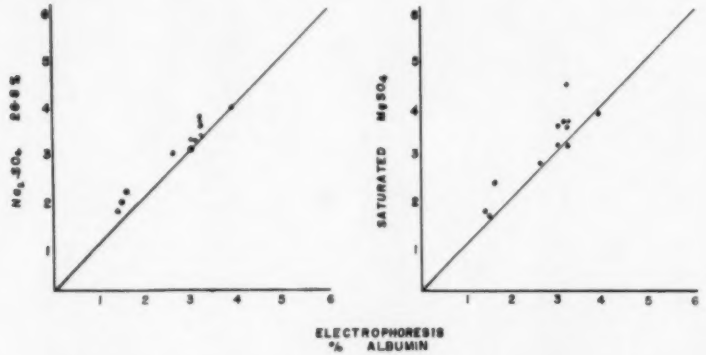

Fig. 3.—Scattergrams illustrate a comparison of plasma albumin values in grams per cent obtained with 26.8 per cent Na_2SO_4 and saturated $MgSO_4$ precipitation methods with respect to electrophoresis values. The solid black line represents 100 per cent correlation for the method indicated.

TABLE II. COMPARISON OF PLASMA ALBUMIN LEVELS OBTAINED WITH 26.8 PER CENT Na_2SO_4 AND SATURATED $MgSO_4$ WITH THE VALUES OBTAINED BY ELECTROPHORESIS

SUBJECTS	TOTAL PROTEIN	ALBUMIN (GM. PER CENT)		
		Na_2SO_4 37° C. 26.8 PER CENT	$MgSO_4$ 37° C. SATURATED	ELECTROPHORESIS
1	7.22	3.48	3.47	3.20
2	7.11	3.61	3.11	3.16
3	5.85	2.12	2.34	1.57
4	6.09	3.90	3.82	3.85
5	5.94	1.85	1.62	1.52
6	6.10	1.71	1.56	1.42
7	6.53	3.26	3.47	3.02
8	7.87	3.26	3.59	3.16
9	6.56	3.19	3.55	3.13
10	6.63	2.93	2.68	2.57
11	7.55	3.66	4.36	3.24
12	6.23	2.94	3.10	3.00
Average	6.64	2.99	3.05	2.73
S.D.		0.69	0.82	0.76

Table II represents the data obtained from plasma of 12 additional hospital patients. The methods used were the proposed method and the saturated solution of $MgSO_4$.[8]

In Milne's method[9] precipitation of the globulins is carried out in the incubator at 37° C. for either 6 hours or for overnight. Filtration is carried out in the incubator and the clear filtrate analyzed for albumin. Kingsley[4] proposes vigorous shaking of the albumin-sulfate (22 per cent) and ether mixture and separation of globulins by centrifugation. The proposed method combines the features of both the methods[4, 9] and introduces an additional important step necessary for an accurate, readily reproducible albumin determination. This step calls for gentle inversion of plasma-sulfate-ether mixture in an ordinary test tube by a specific number (11) of inversions for the purpose of precipitation of globulins.

Jager and associates[14] have discovered that in the $MgSO_4$ method described by Popják and McCarthy,[8] shaking of the serum-salt mixture results in lowering of the albumin values; increased shaking results in further lowering of the albumin values. They have demonstrated this by subjecting albumin-salt solution containing 4.9 Gm. per cent of albumin to shaking for increasing intervals of time. The concentration of albumin in the filtrate decreased progressively. After 24 hours of shaking only 0.9 Gm. per cent of albumin remained in solution. Saifer and Zymaris[15] have also shown a similar observation with Na_2SO_4 and Na_2SO_3. They point out that the degree of precipitation of the globulins from the plasma-salt-ether mixture is effected by the air space above the ether layer. Greater volume permits more intense agitation which then results in greater protein precipitation.

It is a practice in many clinical laboratories to precipitate the globulins by shaking the plasma-salt mixture an unspecified number of times that may not be uniform from sample to sample. This may result in values that cannot be duplicated. The proposed modification will simplify and standardize the details for accurate albumin solution preparation. The close agreement with the electrophoresis albumin values (Table I and Table II) justifies adaptation of this procedure.

SUMMARY

A modified salting-out procedure for separation of plasma albumin is described which eliminates a source of error that may result from excessive shaking of the plasma-salt mixture.

Albumin values obtained with this proposed method are in good agreement with the values obtained by electrophoresis.

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An Editorial

Advance

An *abnormal* serum reference standard has been announced.

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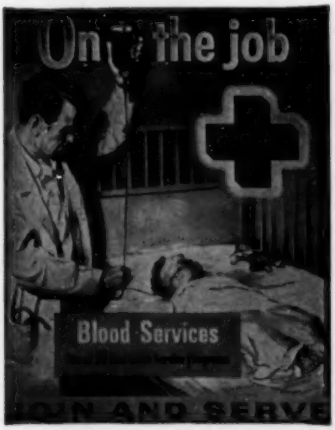

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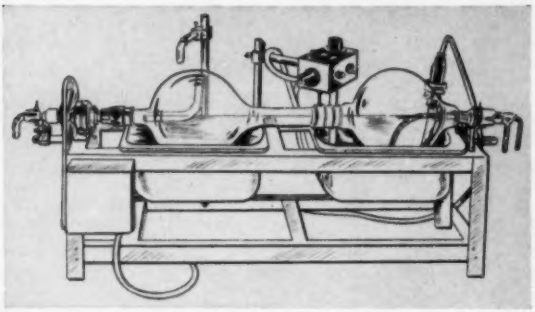

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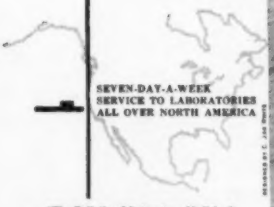

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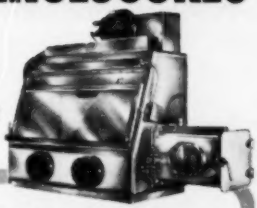

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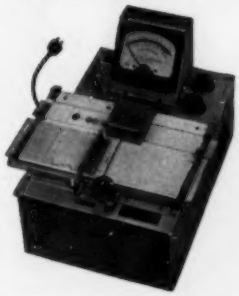

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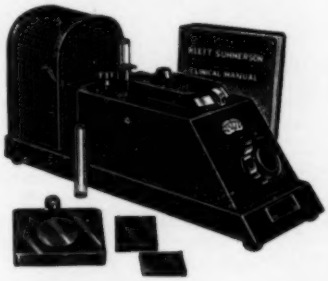

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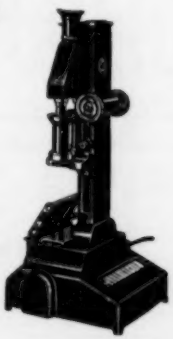

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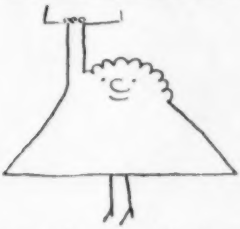

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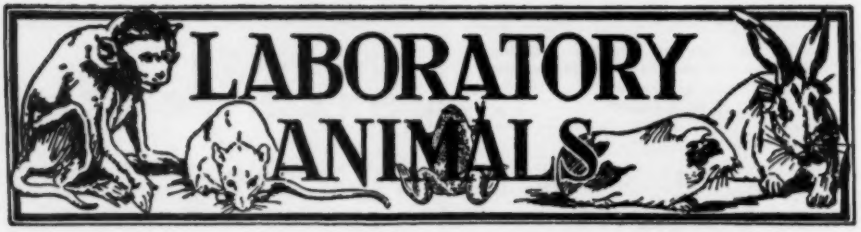

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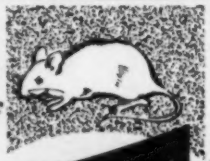

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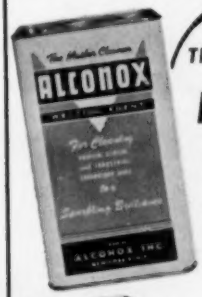

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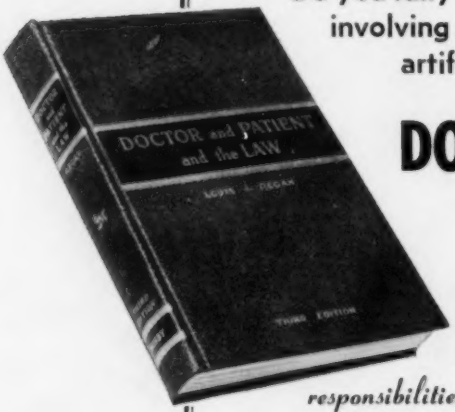

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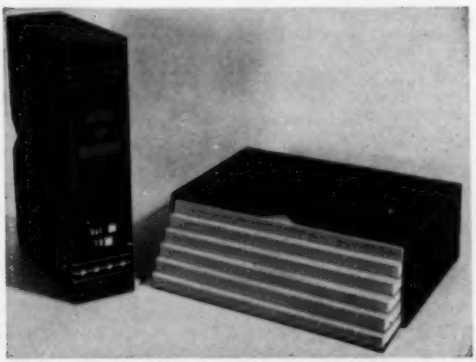

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